(54) Title: Vpr AND Vpx PROTEINS OF HIV

(57) Abstract

   This invention relates to a biologically active peptide fragment of the Vpr protein of human immunodeficiency virus, to pharmaceutical compositions comprising these peptides or biologically active analogues thereof, to antagonists of the peptides, and to pharmaceutical compositions comprising these antagonists and to therapeutic and screening methods utilising compounds and compositions of the invention. In one preferred embodiment, the invention provides an antagonist of the Vpr protein of human immunodeficiency virus (HIV), or of a biologically active fragment or analogue thereof, comprising at least one amino acid sequence motif selected from HFRIG and HSRIG which has the ability to inhibit one or more activities mediated by Vpr, selected from the group consisting of growth arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption, and effects on the endoplasmic reticulum. The invention also relates to use of Vpr protein, or a biologically active fragment or analogue thereof comprising the consensus sequence, in treatment of conditions mediated by cellular proliferation or caused by eukaryotic pathogens.

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Vpr and Vpx proteins of HIV

This invention relates to a biologically-active peptide fragment of the Vpr protein of human immunodeficiency virus, to pharmaceutical compositions 5 comprising these peptides or biologically-active analogues thereof, to antagonists of the peptides, and to pharmaceutical compositions comprising these antagonists and to therapeutic and screening methods utilising compounds and compositions of the invention.

10   Background of the Invention

HIV-1, the causative agent of AIDS, is a complex retrovirus like other primate lentiviruses, having genes *tat, rev, vif, vpr, vpu,* and *nef* that are not found in simple retroviruses. While the functions of *tat* and *rev* 15 are fairly well understood, the remainder, often referred to as auxiliary genes because they are not essential for *in vitro* infectivity of the virus, have poorly understood roles in pathogenesis.

HIV-1 viral protein R (Vpr) (Wong-Staal *et al,* 20 1987) is a virion-associated protein (Cohen *et al,* 1990a; Yuan *et al,* 1990). There have been reports that HIV-1 Vpr is a weak transcriptional activator (Ogawa *et al,* 1990; Cohen *et al,* 1990b) and that it binds to the HIV-1 Gag protein (Lu *et al,* 1993; Paxton *et al,* 1993; Lavallee 25 *et al,* 1994). Although Vpr is not essential for virus replication in established cell lines (Dedera *et al,* 1989; Cohen *et al,* 1990b), there is evidence to suggest that it may have a critical function for viral replication in primary macrophages (Balotta *et al,* 1993; Matsuda *et al,* 30 1993). Because of its association with the virion, it has been suggested that Vpr has an early role in HIV-1 infection, possibly in penetration or uncoating of the virus (Cohen *et al,* 1990a; Yuan *et al,* 1990; Yu *et al,* 1990).

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Vpr is one of the most highly conserved proteins of HIV-1, and exists as Vpr and/or Vpx in all primate lentiviruses (Tristem et al, 1990; see Fig. 4A). Vpx is similarly virion-associated (Cohen et al, 1990a; Yuan

5   et al, 1990; Yu et al, 1988, 1990, 1993). HIV-2 Vpr is essential for productive infection of human macrophages (Hattori et al, 1990), but like HIV-1 Vpr it is dispensible for replication in established cell lines (Dedera et al, 1989). Similarly HIV-2 Vpx is dispensible in established

10   cell lines (Yu et al, 1988; Guyader et al, 1989; Hu et al, 1989) but is required for infection in fresh macrophages (Guyader et al, 1989; Yu et al, 1991), and augments viral infectivity in peripheral blood lymphocytes (Kappes et al, 1991). Perhaps most convincing of all, it has been

15   observed that there is a drive in vivo for retention of an intact vpr reading frame and that mutations in vpr lead to a low virus burden in Rhesus monkeys (Lang et al, 1993).

We have cloned the Vpr gene in yeast, and have compared the effect of Vpr protein on haploid yeast cells

20   with the effects of the proteins Vif, Vpu and Nef. We have surprisingly found that the Vpr protein has profound effects on cell growth, while the other proteins tested have no effect. The Vpr protein causes growth arrest, and this appears to be mediated by effects on the cytoskeleton.

25   We have identified the portion of the Vpr protein which is critical for this activity. This critical portion comprises a conserved amino acid sequence motif, H(S/F)RIG, and peptides comprising this portion are active when added extracellularly to mammalian or yeast cells. Since the Vpr

30   protein appears to have an early and possibly critical role in HIV infection, it represents a useful therapeutic target.

We have also found that the Vpr protein, and particularly the C-terminal sequence thereof, has a general

35   antiproliferative effect on eukaryotic cells, and therefore is useful in the treatment of conditions mediated by cell proliferation.

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Summary of the Invention

According to one aspect, the invention provides a
method of treatment of HIV infection, comprising the step
of administering to a subject in need of such treatment an
5   effective amount of an antagonist of Vpr protein, or of a
biologically active fragment or analogue thereof comprising
at least one motif selected from HFRIG and HSRIG, thereby
to prevent HIV infection, to prevent progression of HIV
infection to symptomatic AIDS, or to alleviate the symptoms
10   of AIDS.

Preferably the Vpr protein comprises at least one
sequence selected from the group consisting of HSRIG,
HFRIG, HSRIS, HFRAG, HIRAG, HLRAG, RSRKG, RSRIS and RSRIG,

In a second aspect, the invention provides an
15   antagonist of the Vpr protein, or of a biologically active
fragment or analogue thereof, as defined above, which has
the ability to inhibit one or more activities mediated by
Vpr, selected from the group consisting of growth arrest,
cell replication arrest, cytotoxicity, cytoskeletal
20   disruption, and effects on the endoplasmic reticulum. It
is considered that such antagonists will be useful as
therapeutic agents for treatment of HIV infection.

The invention also encompasses a pharmaceutical
composition comprising as active component an antagonist of
25   Vpr as defined above, together with a pharmaceutically-
acceptable carrier.

The person skilled in the art will recognise that
specific antibody, preferably monoclonal antibody, directed
against Vpr or a biologically active fragment or analogue
30   thereof, and antisense RNA or triple-stranded DNA which
prevents expression of Vpr or of said biologically-active
fragment or analogue, provide methods of inhibition of the
activity of Vpr, and consequently are within the scope of
this invention. Methods for production of monoclonal
35   antibodies against a given peptide sequence, and methods
for inducing antisense RNA or triple-stranded DNA
production in a target cell are well known in the art. For

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example, a *vpr* gene in which the region encoding C-terminal
portions of the Vpr protein has been replaced by an
inhibitory antisense sequence or by a sequence which
encodes an inhibitory peptide could be used for gene
5   therapy of HIV infection or of AIDS.

In a third aspect, the invention provides a
method of screening compounds suspected of being useful as
antagonists of Vpr protein , or of a biologically active
fragment or analogue thereof as defined above, comprising
10   the step of measuring the effectiveness of a test compound
in inhibiting the activity of Vpr in an assay of a
biological activity selected from the group consisting of
growth arrest, cell replication arrest, cytotoxicity,
cytoskeletal disruption, and effects on the endoplasmic
15   reticulum, as herein described.

Our results indicate that the Vpr protein or
biologically active fragment or analogue thereof has
activities which can be attacked at either the
intracellular or extracellular level, and therefore both
20   types of biological activity are within the scope of the
invention.

According to a fourth aspect, the invention
provides a vaccine for prevention of HIV infection or for
alleviation of the effects of HIV infection, comprising
25   human immunodeficiency virus-1 or human immunodeficiency
virus-2 from which the portion of the HIV genome encoding
at least the C-terminal 21 amino acids of the Vpr sequence
has been deleted, together with a pharmaceutically-
acceptable carrier. Preferably the portion of said genome
30   encoding at least the C-terminal 33 amino acids of the Vpr
sequence has been deleted. Even more preferably both a
portion of the genome encoding the C-terminal region of the
Vpr sequence and the portion of the HIV genome encoding the
N-terminal of the Nef gene have been deleted. Relevant
35   portions of the Nef gene are described in our co-pending
Patent Application No. PCT/AU94/00254 (WO 94/26776), filed
18 May 1994.

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According to a fifth aspect, the invention
provides a method of treatment of a disease mediated by
cell proliferation, comprising administration to a mammal
in need of such treatment of an effective amount of Vpr
5   protein, or of a biologically active fragment or analogue
thereof comprising at least one sequence of the consensus
sequence disclosed herein, thereby to inhibit proliferation
of cells mediating the said disease.

Diseases mediated by cell proliferation include,
10   but are not limited to, cancer, leukaemias and psoriasis.

Proliferating cells are highly calcium dependent.
We have found that the anti-proliferative effect of Vpr
peptides is blocked by an inhibitor of Ca^{2+}-channel
transport. Therefore in this aspect of the invention, the
15   Vpr protein or fragment or analogue thereof may optionally
be used in conjunction with an enhancer of Ca^{2+}-channel
transport.

According to a sixth aspect, the invention
provides a method of treatment of a disease caused by a
20   pathogen, comprising the step of administering to a mammal
in need of such treatment of an effective amount of Vpr
protein, or of a biologically-active fragment or analogue
thereof comprising at least one sequence of the consensus
sequence disclosed herein. Diseases caused by bacteria,
25   parasites, yeasts, or fungi are all within the scope of
this aspect of the invention.

According to a seventh aspect, the invention
provides an agent for delivery of a pharmaceutically active
substance to a cell membrane or to the interior of a cell,
30   comprising a Vpr peptide or a biologically active fragment
or analogue thereof comprising at least one amino acid
sequence motif selected from HFRIG and HSRIG, said peptide,
fragment or analogue being linked to said pharmaceutically
active substance.

35   The invention also provides a method of delivery
of a pharmaceutically active substance to a cell membrane
or to the interior of a cell, comprising the step of

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contacting said cell with an agent as defined above.

    The active substance may be any chemical entity capable of being linked to a peptide, and in particular may be a peptide or a nucleotide. Coupling may be effected by any convenient means, for example chemical coupling using agents such as carbodiimide. Where the active substance is a peptide, the Vpr peptide and the pharmaceutically active peptide may be synthesised together by recombinant means as a fusion protein. The person skilled in the art will be aware of a variety of suitable pharmaceutically active agents which could be delivered in this way, and of methods whereby they may be linked to the Vpr protein. Such a person will also be aware of methods suitable for testing whether the linkage has been effective, and whether the agent retains the desired pharmaceutical activity.

    In preferred embodiments of the invention, the Vpr protein is a fragment comprising at least the C-terminal 21 amino acids of the Vpr sequence, more preferably at least the C-terminal 33 amino acids of the Vpr sequence.

    It will be clearly understood that Vpr protein or its biologically active fragments linked to a carrier or fusion protein, such as glutathione-S-transferase (GST), are within the scope of the invention. It will be further understood that recombinant, synthetic and naturally-derived Vpr protein and fragments and analogues thereof are within the scope of this invention.

    While the description herein relates specifically to Vpr protein of HIV-1, as described above HIV-2 also possesses a Vpr protein, and it will be clearly understood that the invention is equally applicable to the Vpr protein and the equivalent of the H(S/F)RIG motif derived from HIV-2, and to the Vpx protein of other lentiviruses.

    Throughout this specification, the single letter code of abbreviations for amino acids is used. S/F indicates that either S or F may be present.

Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the drawings, in which:

5   Figure 1 illustrates the scheme employed for cloning of the *vpr* gene for expression in yeast;

Figure 2 shows the effect on growth of expression of HIV-1 auxiliary proteins in yeast;

Figure 3 shows the morphological changes in yeast
10   cells expressing Vpr.

Copper-induced yeast cells were examined by light microscopy. A DY150 [pYEULCBX] control transformant is shown in the top panel (A) and a typical large DY150 [pYEULCBX.Vpr] transformant is shown on the bottom panel
15   (B). The bar represents 10 μm.

Figure 4 shows the results of analysis of induced cells by flow cytometry;

Figure 5 shows the identification of the toxic region in Vpr;

20   A series of Vpr constructs is indicated. The region of Vpr produced by the construct is represented by pale blocks, while dark blocks represent GST; GST is not drawn to scale. The position of *BamHI* (B), *EcoRI* (E) and *SalI* (S) sites relative to the protein sequence is
25   indicated. H(S/F)RIG, shown in bold, is encoded by sequences on either site of the *SalI* site. Growth of the transformed yeast cells producing these proteins is recorded in the right column.

Figure 6 shows the relationship between HIV-1 Vpr
30   and proteins from HIV-2, SIV and yeast.

A.   Alignment of HIV-1 Vpr with Vpr relatives.

Vpr and Vpx proteins are aligned in their entirety. Sequences are derived from HIV-1 Vpr NL4-3 (Adachi *et al*, 1992), HIV-2ROD (Clavel *et al*, 1992) and
35   SIVmac239 (Regier and Desrosiers, 1990). Regions with three or more identical amino acids are shaded.

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B.   Alignment of Sac1p and HIV-1 Vpr.

The entire amino acid sequence of Vpr is aligned with amino acids 77-176 of Sac1p (Cleves et al, 1990). Identical residues are shaded, and the residues in the H(S/F)RIG motif are underlined.

Figure 7 illustrates the osmosensitivity of yeast expressing Vpr.

Peptide 1	NH_2-VTRQRRARNGASRS-COOH
Peptide 2	NH_2-CRHSRIGVTRQRRARNGASRS-COOH
Peptide 3	NH_2-HFRIGCRHSRIGVTRQRRARNGASRS-COOH

Figure 8 illustrates the strategy used for the construction of mutant viruses.

Figure 9 shows the replication kinetics of mutant viruses in human PBMC, as measured by reverse transcriptase assay.   A. Stimulated cells; B. Unstimulated cells

Figure 10 shows the effect of synthetic Vpr peptides on yeast colony formation.   A, no peptide; B, Peptide 1;   C, Peptide 2;   D, Peptide 3.

Figure 11 summarises the results of the response of yeast cells to synthetic Vpr peptides.   Treatment conditions are as described in Example 11.

Figure 12 shows the dose response relationship for Peptide 3.   Peptide 3 was added to yeast cells over a concentration range and cells were assayed for colony formation.

Figure 13 shows the effect of yeast cell concentration on colony formation in the presence of a Vpr peptide.   Various cell concentration were incubated in the presence of 5 μM Peptide 3 and cells were assayed for colony formation.

Figure 14 shows the results of flow cytometric analysis of propidium iodide (PI) uptake following incubation of yeast cells with synthetic Vpr peptides for 1 h.   Samples are No peptide, Peptide 1, Peptide 2 and Peptide 3.

Figure 15 shows the results of flow cytometric analysis of propidium iodide uptake following incubation of yeast cells with Peptide 3 for various times.

Figure 16 shows the results of flow cytometric analysis of propidium iodide uptake in RC2a cells electroporated with synthetic Vpr peptides.

Figure 17 shows results obtained when mammalian cells were electroporated and analysed by flow cytometry for changes in cell structure as measured by forward and side scatters.

Figure 18 shows protection of yeast cells by TMB-8.

Figure 19 shows the association of FITC (fluorescein isothiocyanate)-labelled peptides with CD4$^+$ human cells measured by flow cytometry following (A) electroporation, and (B) extracellular addition without electroporation.

Figure 20 shows the association of FITC-labelled peptides without electroporation in *S. cerevisiae* yeast cells measured by flow cytometry.

Figure 21 shows the internalised FITC-labelled peptide 3 in human CD4$^+$ cells (A) and in yeast cells (B).

Figure 22 shows the genetic interaction between Vpr and Sac1p and actin.

Yeasts and bacteria

Yeast strains employed in this study were *Saccharomyces cerevisiae* strain DY150 (*MATa ura3-52 leu2-3, 112 trp1-1 ade2-1 his3-11 can1-100*), *Candida albicans* clinical isolate JRW5, *Candida glabrata* strain L5 (*leu*), *Kluyveromyces lactis* strain MW-98-8c (*α uraA arg lys*) and *Schizosaccharomyces pombe* strain SpULA (*ade6-704 ura4-D18 leu1-32*)h⁻. Strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). An *Escherichia coli* strain TG1 Δ(*lac-proAB*) *supE thi hsdΔ5 F'*[*traD36 proAB⁺ lacI^q lacZΔM15*] was also employed for toxicity studies and plated onto 2xYT medium (1.6% tryptone, 1% yeast extract, 0.5%

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NaCl).

Mammalian cells

       Two CD4+ cell lines, RC2a and Jurkat, were
maintained in RPMI-1640, containing 10% heat inactivated
5   foetal calf serum (HIFCS). Mononuclear cells were isolated
from HIV-1 seronegative blood obtained from blood bank
volunteers by a standard Ficoll/Hypaque density gradient
method. The peripheral mononuclear leukocyte cells (PBMC)
were stimulated with phytohaemagglutinin (PHA : 10 μg/10^6
10   cells) for 48 h at 37°C, and were washed and resuspended in
IL-2 medium, containing RPMI-1640 medium with 10% HIFCS,
10% recombinant human Interleukin-2 (Boehringer Mannheim),
5 mM Hepes, 0.1% sodium bicarbonate, 25 μg/ml glutamine,
100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml
15   polybrene (Sigma) and 1:1000 anti-interferon (Miles).

Example 1   Molecular Cloning of HIV Genes

       The HIV-1 genomic clone pNL4-3 was used as the
source of HIV-1 genes for amplification by polymerase chain
reaction (PCR). pNL4-3 (Adachi et al, 1986) was obtained
20   from the National Institutes of Health (NIH) AIDS Research
and Reference Reagent Program, National Institutes of
Allergy and Infectious Diseases, NIH (Adachi et al, 1986).
The cloning of the vpu and nef genes has been described
previously (Macreadie et al, 1992, 1993). The scheme used
25   for cloning of vpr is described in Figure 1, while the
cloning of vif employed PCR and similar known procedures.
vpr was amplified from the HIV-1 genomic clone pNL4-3
(Adachi et al, 1986) using PCR and the primers shown. The
PCR product was cleaved with BamHI + SmaI and cloned into
30   the yeast - E. coli shuttle vector pYEULCBX (Macreadie et
al, 1992), and digested with BamHI + EcoRI (T4 polished) to
produce pYEULCBX.Vpr. In like fashion the other HIV-1
genes, nef, vpu and vif were also cloned into pYEULCBX to
produce pYEULCBX.Nef27 (Macreadie et al, 1993),
35   pYEULCBX.Vpu (Macreadie et al, 1992) and pYEULCBX.Vif.

These plasmids were designed to direct the copper-inducible production of Nef, Vpu and Vif, respectively. *vif* primers were 5' GCTCC<u>GGATCC</u>*ATG*GAAAACAGATGGCAGG and 5' CG<u>CCCGGG</u>AGCTCTA*AAA*GCTCTAGTGTCC. The *vif* PCR product was cloned as a *Bam*HI-*Sma*I fragment. *Bam*HI and *Sma*I cloning sites in the primers (above) are underlined, while sequences in italics represent the *vif* start and stop codons. All amplified DNA was sequenced to verify the absence of errors. Cloning of *nef, vpr, vpu* and *vif* genes into the yeast expression vector pYEULCBX was designed to direct the copper-inducible production of Nef, Vpu and Vif, respectively.

Example 2   Endogenously-Expressed *vpr* Protein Causes Growth Arrest in Yeast

In this study we expressed *vpr* in yeast in order to discern its functions. At the same time, as part of a general examination of the effects of the HIV-1 regulatory proteins on simple cellular functions, we also produced Vif, Vpu and Nef in haploid yeast and looked for their effects on cell growth. This was achieved by cloning *vpr* and the genes of other HIV-1 auxiliary proteins into the expression vector pYEULCBX to produce pYEULCBX.Vpr (see Fig. 1), pYEULCBX.Nef27 (Macreadie *et al*, 1993), pYEULCBX.Vpu (Macreadie *et al*, 1992), and pYEULCBX.Vif (this study).

Strain DY150 (*MATa ura3-52 leu2-3,112 trp1-1 ade2-1 his3-11 can1-100*; Macreadie *et al*, 1993), obtained from Dr David Stillman at the University of Utah Medical Center, was transformed with the above yeast vectors plus vectors for the copper-inducible production of glutathione S-transferase (GST) and GST fused to Vpr.DY150 was grown on YEPD medium (1% yeast extract, 2% peptone, 2% glucose). Yeast cells were transformed by the electroporation procedure of Becker and Guarente (1991) and transformants were grown on minimal selective medium containing 20 µg/ml histidine, adenine and tryptophan and solidified, when

required, with 3% Phytagar™ (Gibco). Expression was
induced by the addition of $CuSO_4$ to the amounts indicated,
and growth was assayed. Transformants were suspended in
sterile water and dropped out on to plates for growth at

5   28°C. The results are illustrated in Figure 2, which shows
SD plates (0.67% yeast nitrogen base (Difco), 2% glucose)
containing 0.25 mM $CuSO_4$, 20 μg/ml histidine, adenine and
tryptophan and solidified with 3% Phytagar™ (Gibco). The
proteins produced by the transformants are indicated.

10   As shown in Figure 2, profound effects on cell
growth were caused by the Vpr protein, while the other
HIV-1 proteins tested had no effect on vegetative cell
growth. Low levels (0.25 mM) of $CuSO_4$ caused total growth
arrest in cells expressing Vpr (Fig. 2), while no adverse

15   effects were caused by the other proteins even with
induction levels as high as 1 mM $CuSO_4$. The effect of Vpr
was unrelated to copper toxicity, since with no added
$CuSO_4$, where basal expression from the *CUP1* promoter is 5%
of the induced level (reviewed in Macreadie *et al*, 1994),

20   Vpr transformants grew at a slower growth rate than control
transformants.

              The Vpr toxicity was found to be due to growth
arrest, not killing, since induced cells, even after 24
hours in the presence of the inducer, formed colonies when

25   plated on media with no added copper. The DY150
[pYEULCBX.Vpr] transformant colonies grown up from the
assay were considerably smaller than DY150 [pYEULCBX]
transformant colonies. These "small" colonies grew like
the parental DY150 [pYEULCBX.Vpr] transformant upon

30   subsequent culture without added copper (data not shown),
indicative of a cell cycle arrest after induction of Vpr
synthesis followed by eventual recovery and return to the
normal cell cycle.

       Example 3   Arrested Cells are Greatly Enlarged
35   Examination of cells by light microscopy
indicated that induced cells producing Vpr had a grossly

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altered morphology. As shown in Figure 3, Vpr-producing
cells had a diameter of 16 μm, more than twice the diameter
of the control DY150 [pYEULCBX] transformants grown under
the same conditions. It appeared that most of the
5   intracellular space in the large cells was devoid of
structure and occupied by a single large organelle,
possibly a vacuole.   This suggests that the
DY150[pYEULCBX.Vpr] transformants were arrested in growth
before cell division.

10   Example 4   Flow Cytometry Analysis
          Cells were analysed and sorted using a Coulter
Epics® Elite flow cytometry. Illumination was with a 488
nm Argon ion laser, and forward angle light scatter
(related to cell size) and side scatter were recorded.
15   Cells were sorted on the basis of forward angle light
scatter. Live cells were gated by propidium iodide
exclusion, indicated by absence of fluorescence emission at
greater than 600 nm following staining with 2 μg/ml
propidium iodide.

20   Induced yeast cells were analysed by flow
cytometry forward angle light scatter (proportional to cell
size) in order to assess the proportion of altered cells.
The results are shown in Figure 4, and confirmed that in
the cell population Vpr transformants exhibited a greater
25   degree of forward light scattering, indicative of their
larger size. Populations, containing over 50,000 cells,
are for DY150 [pYEULCBX] and DY150 [pYEULCBX.Vpr] as
indicated.

     Example 5   Location of Sequences Causing Growth Arrest
30   Includes H(S/F)RIG Repeated Motifs
          The sequences responsible for causing the growth
arrest were identified by testing various portions of the
Vpr protein for effects on cell growth. Since Vpr fused to
glutathione S-transferase (GST) also caused a growth arrest
35   (Fig. 2; construct GST-Vpr, Fig. 5), we also produced a

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series of GST fusion proteins in the yeast GST-fusion
vector, pYEULCGT (Ward et al, 1994).

Deletion of the last 33 amino acids of Vpr,
encoded by an EcoRI fragment (constructs VprBE and
5   GST-VprBE, Fig. 5), relieved the growth arrest, while the
addition of this portion of Vpr to GST (construct
GST-VprEE, Fig. 5) caused a growth arrest, indicating that
this domain was responsible for the growth arrest. A
partial growth arrest was also seen with the addition of
10   just the last 21 amino acids of Vpr to GST (construct
GST-VprSE, Fig. 5).

In each case the growth arrest correlated with
cell enlargement, as judged by flow cytometry analysis and
light microscopy. Significantly, this C-terminal sequence
15   is the region lacking in many laboratory HIV-1 isolates
that encode a truncated vpr gene product of 73 amino acids
due to a T insertion (Yuan et al, 1990; Ogawa et al, 1990;
Lavallee et al, 1990). The Vpr in these isolates does not
associate with virions (Ogawa et al, 1990), presumably
20   because of the truncation. Our findings confirm the
importance of the same C-terminal region, but for another
reason. This growth arrest in yeast may be linked to AIDS
pathogenesis.

The region of HIV-1 Vpr that causes cell growth
25   arrest has been compared with known Vpr relatives, the
closest relative being the SIV Vpr followed by HIV-2 Vpr,
and then Vpx proteins (Fig. 6A). The sequence comprises
33% arginine, a much higher arginine content than that
found in comparable portions of Vpx proteins. It is
30   notable that there is conservation of a repeated motif,
H(S/F)RIG, in Vpr species. The motif is present at amino
acids 72-75 (encoded in the EcoRI-SalI fragment), and at
amino acids 78-82 (encoded in the SalI-EcoRI fragment).
The greater toxicity caused by the fragment encoding two
35   copies may indicate a copy number effect or possibly a
conformational effect.

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In a search for a cellular relative to Vpr using the program ALIGN, we found that a yeast protein, Sac1p (Cleves *et al*, 1989), has the most significant sequence similarity of cellular proteins listed in the Genbank database (release 82.0). In the alignment of Sac1p and Vpr (Figure 6B) it can be seen that Sac1p has 60% identity in the H(S/F)RIG motifs including the terminal Gs, the part of the motif that is totally conserved in Vpx as well. Over the entire alignment there are 32% identical and 45% similar amino acids.

Example 6   Peptide Synthesis

The peptides produced were as follows:

Peptide 1   NH₂-VTRQRRARNGASRS-COOH

Peptide 2   NH₂-CRHSRIGVTRQRRARNGASRS-COOH

Peptide 3   NH₂-HFRIGCRHSRIGVTRQRRARNGASRS-COOH

Peptide 4   NH₂-HFRIGCRHSRIG-COOH

Peptide 5   NH₂-RHSRIGVTRQRRARNGASRS-COOH

Peptide 6   NH₂-IFRAGTRYFRRG-COOH

Peptides were synthesised on an Applied Biosystems 430A Peptide Synthesizer, using the FastMoc solid-phase technique in which α-amino groups were protected by base-labile Fmoc (9-fluorenylmethyloxy-carbonyl) groups. The shortest sequence was synthesised on to the resin, then approximately one third of the peptide/resin was removed from the reaction vessel. Synthesis was then continued on the remainder until the second peptide was assembled, at which stage half of the peptide/resin was removed from the reaction vessel. The third peptide was then assembled on to the remaining peptide/resin.

The arginine side chains were protected by *tert*-butyl groups and glutamic acid by the O-*tert*-butyl group. Couplings were achieved by using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium

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hexafluorophosphate activation of amino acids and
N-methylpyrrolidone as solvent. The peptides were cleaved
from the resin with trifluoroacetic acid (plus phenol,
ethanedithiol, thioanisole and water as scavengers). The
5   peptides were dialysed against electroporation buffer
(0.213 g/l Na_2HPO_4, 0.068g/l KH_2PO_4, 93.1 g/l sucrose)
(Wojchowski and Sytkowski, 1986) before electroporation.

       For Peptides 4 to 6, protection was as follows:
α-amino groups by base-labile 9-fluorenylmethloxycarbonyl
10   (Fmoc) groups; arginine side chains by 2,2,5,7,8-
pentamethylchroman-6-sulfonyl (Pmc); serine and threonine
by tert-butyl groups; asparagine, glutamine, histidine and
cysteine by trityl; and glutamic acid by the O-tert-butyl
group. Couplings were achieved by using 2-(1H-
15   benzotriazol-1-yl)-1,1,3,3-tetramethyluonium
hexafluorophosphate (HBTU) activation of amino acids and N-
methylpyrrolidone (NMP) as solvent. The peptides were
cleaved from the resin with TFA, plus phenol,
ethanedithiol, thioanisole and water as scavengers.

20   Example 7   H(S/F)RIG Motifs in Synthetic Peptides
                       Cause Osmosensitivity
       We further investigated the function of the
H(S/F)RIG motifs using the synthetic peptides:

                    NH_2-VTRQRRARNGASRS-COOH
25   NH_2-CRHSRIGVTRQRRARNGASRS-COOH
           NH_2-HFRIGCRHSRIGVTRQRRARNGASRS-COOH

produced in Example 6, that contain the penultimate 14, 21
and 26 amino acids, respectively, of Vpr. The H(S/F)RIG
motif (underlined) is present at zero, one and two copies,
30   respectively, within these peptides. These peptides were
electroporated into yeast cells which were then analysed
for osmosensitivity. Peptides, dissolved in
electroporation buffer at 2 mg/ml, were electroporated into
yeast cells using a Baekon 2000 (Saratoga, CA). Conditions

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for the treatment in the Baekon 2000 were: 2^{11} pulses, 8
kV, 0.8 sec burst time, 100 μsec pulse time, 10 cycles, 1
mm gap between solution and upper electrode. The cuvettes
contained 30 μl of yeast suspension in fresh YEPD growth

5   medium plus 5 μl of Dulbecco's Phosphate-Buffered Saline
and 5 μl of peptide solution. It was found necessary to
achieve a kill of 60-80% in order to achieve uniform
penetration of the surviving cells.

        Cells were examined for osmosensitivity by
10   plating onto YEPD medium and YEPD medium containing 1.2 M
KCl, 1.8 M sorbitol or 0.9 M NaCl, and counting the numbers
of colony-forming units, as described by Chowdhury et al
(1992). Osmosensitivity was calculated by comparing the
relative numbers of colony-forming units on the two media.
15   All viable cells, including osmosensitive cells, grew on
YEPD, but those that were osmosensitive did not grow on
high osmotic strength media. The results, presented in
Figure 7, show that cells treated with the peptide lacking
an H(S/F)RIG motif were essentially unperturbed. However,
20   the peptides containing H(S/F)RIG motifs caused osmotic
sensitivity such that up to 50% of the cells were killed on
high osmotic strength media. The effects were commensurate
with the number of copies of H(S/F)RIG motif present,
indicating a direct role for this sequence.

25   Example 8   Pathogenicity is Associated With the
                          Sequence Containing H(S/F)RIG Motifs
        The region of the Vpr protein containing
H(S/F)RIG motifs may be correlated to the pathogenicity of
human and simian immunodeficiency viruses. A brief
30   compilation of sequences of Vpr and Vpx from human and
simian immunodeficiency viruses is shown in Table 1. There
is almost total conservation of the 12 amino acids
containing two repeated H(S/F)RIG motifs in HIV-1, a highly
pathogenic virus.

35   Seven simian immunodeficiency virus Vpr sequences
show high conservation (two changes) of the sequence

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containing the H(S/F)RIG motifs. However, the two
sequences shown by the asterisk have poor conservation of
the sequence (8 or 9 changes). Both the mandrill virus and
the Sykes' monkey virus show poor sequence conservation,
5   and are reported to cause asymptomatic infection (Hirsch et
al, 1993; Tsujimoto et al, 1989).

In HIV-2 isolates there are between two and five
changes from the reference sequence. HIV-2 is less
pathogenic than HIV-1, and we believe that these changes
10   may be a reason for the reduction in pathogenicity.
Additionally the presence of Vpx may reduce pathogenicity.
Matsuda et al (1993) showed that when Vpx replaced Vpr in
HIV-1, the virus lost its infectivity. Thus we predict
that any virus that produces Vpx may be expected to be less
15   pathogenic than one which produces Vpr alone.

Table 1
H(S/F)RIG Motifs in Vpr Relatives

HIV-1

NL43	HFRIGCRHSRIG
HAN	HFRIGCRHSRIG
MN	HFRIGCRHSRIG
ELI	HFRIGCQHSRIG
SC	HFRIGCRHSRIG
LAI	HFRIGCRHSRIG
SF2	HFRIGCQHSRIG
MAL	HFRIGCQHSRIG
OY1	HFRIGCQHSRIG
NDK	HFRIGCQHSRIS
NH52	HFRIGCQHSRMG

consensus   HFRIGCRHSRIG
                      L   Q   MS

SIV

SIVmac239	HFRGGCIHSRIG
SIVmac142	HFRSGCSHSRIG
SIVmac251	HFRGGCNHSRIG
SIVmacMNE	HFRGGCTHSRIG
SIVmmm H4	HFRSGCAHSRIG
SIVmmmPBJ	HFRGGCRHSRIG
SIV cpz	HFRLGCQHSRIG

Table 1 (cont)


```
         consensus        HFRGGCRHSRIG
                             S    I
                             L    S
    5                             T
                                  A
                                  Q
                                  N


         SIVmndGB1        HLAQGCDGTFRE *
   10    SIVsykes         HFAAGCPQRTRY *


         HIV-2

         ROD              HFRAGCGHSRIG
         D205             HFRAGCGHSRIG
         ISY              HFRAGCGHSRIG
   15    NIHZ             HFRAGCGHSRIG
         CAM2             HFRAGCNHSRIG
         D194             HIRAGCDRSRKG
         GH1              HLRAGCNRSRIS
         ST               HFRAGCGRSRIG
   20    BEN              HFRAGCNRSRIG

         consensus        HFRAGCGHSRIG
                            I        DR   KS
                            L        N


         Vpx

   25    SIVmac239        HCKKGCRCLGEG
         SIVmac142        HCKKGCRCLGEG
         SIVmac251        HCKKGCRCLGEG
         SIVmacMNE        HCKKGCRCLGEG
         SIVmmm H4        HCKKGCRCLGEE
   30    SIVmmmPBJ        HCKKGCRCLGGE

         consensus        HCKKGCRCLGEG
                                      GE

         HIV2  ROD        HVRKGCTCLGRG
         HIV2  D205       HYTKGCRCLQEG
   35    HIV2  CAM2       HFKRGCTCLGGG
         HIV2  ISY        HFKKGCTCRGEG
         HIV2  NIHZ       HAKRDGTCLGGG
         HIV2  D194       HFKKGCTCLGRG
         HIV2  GH1        HFKRGCTCLGGG
   40    HIV2  ST         HFKRGCTCLGGG
         HIV2  BEN        HFKRGCTCWGED
```

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Table 1 (cont.)

```
        consensus        HFKKGCTCLGGG
                          YRRD R WQRD
                          VT       R E
5                         A
                          S


        SIVagm155        HFRCGCRRRQPF
        SIVagm 3         HFRCGCRRRQPF
        SIVagmTYO        HFRCGCRRRQPF


10      consensus        HFRCGCRRRQPF
```

The overall consensus for the Vpr sequence, excluding those represented by the asterisk, is:

```
                         HFRIGCRHSRIG
                          I L  QR  MS
15                        L G  N
                             S  I
                                S
                                T
                                A
20                              Q
                                G
                                D
```

In summary, it appears that in the sequence HFRIGCRHSRIG, the residues underlined are invariant in Vpr.

25   F can be I or L; the I can be L, G, S or M; the last G can be S. It should also be noted that the C between motifs is invariant.

Example 9   Replication Kinetics of Mutant Proviruses
Production and titration of virus culture

30   Half clones of the mutant and wild-type HIV DNA were co-transfected to HeLa cells (5×10^6 cells) in T25 flasks, by the Lipofectamine (GIBCO-BRL) method. PBMC (20×10^6 cells) were added 12 h after transfection and the cell free virus production was measured at regular

35   intervals. The supernatant was harvested at maximum production of cell free virus and used as stock virus.

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Titrations of virus stocks were done in 24 well Linbro
plates, and the end point dilution was scored by both
Reverse Transcriptase (RT) activity and visible cytopathic
effect. RT assay in microtitre plates were performed
5   according to standard methods.

Construction of mutant provirus

HIVNL 4.3 molecular clone (Adachi et al, 1986)
was re-cloned as two half fragments into the pKP59 vector
for the point mutation of the initiation codons of the *nef*
10   and *vpr* genes. Mutant proviruses were constructed
according to the procedures described in Figure 8, using
the mutagenesis scheme summarized in Table 2.

Table 2

Mutagenesis Scheme

Gene	Oligo	Mutation position (nt)	Nucleotide changes	Final effect	Clone Into
Nef	N7	8788	ATG to AAG	no nef	pKP3EA
	N8	8829	ATG to ATC	expressed	
Vpr	V2	5559 5565	ATG to GTG CAA to TAA	no vpr expressed	pKP5SE
Vpr	VMM	5770 to 5804	deletion of 36 nucleotides	deletion of H(S/F)RIG motifs	pKP3EA

The HIV-1 molecular clone employed was pNL4-3.
20   Because of instability of the full length clone in *E. coli*,
half-clones were constructed in the low copy vector, pKP59,
and stably maintained in *E. coli*. The 5' sequences were
introduced as a *Stu*I-*Eco*RI fragment while 3' sequences were
introduced as an *Eco*RI-*Avr*II fragment. These half-clones
25   could be appropriately digested (*Xba*I+*Eco*RI fragment for
the 5' clone and *Eco*RI+*Hae*II for the 3' clone) and the cut
DNA introduced into mammalian cells where *in vivo*
recombination restored the wild-type virus. To obtain

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mutant virus appropriate segments of pNL4-3 were cloned
into a phagemid, single-stranded DNA was produced, and
second strand was synthesised in the presence of
oligonucleotides, shown in Table 3, which were designed to
5   introduce specific *vpr* and *nef* mutations.

Table 3
Mutagenic Oligonucleotides

Oligonucleotide	Code
5'-GGA TTT TGC TAT AAG A<u>A</u>G GGT GGC AAG-3'	N7
5'-GTA AGG GAA AGA AT<u>C</u> AGA CGA GCT G-3'	N8
5'-CAG AGG ACA G<u>G</u>T GGA A<u>T</u>A AGC CCC CAG AAG-3'	V2
5'-CTG CAA CAA CTG CTG TTT ATC *GTT ACT CGACAG AGG-3'	VMM

* indicates the site targetted for deletion in *vpr*

Following purification and verification of
15   sequence changes the DNA was sub-cloned into the pKP59-
half-clone, replacing the wild-type sequence with a mutant
sequence. The mutant clones do not express Nef, do not
express Vpr, or do not express either protein.

Infection of PBMC
20   Peripheral blood mononuclear cells were infected
at a 0.01 multiplicity of infection (MOI), and the cell
free supernatants were assayed daily for reverse
transcriptase (RT) production by standard techniques.
             In stimulated PBMC, mutant proviruses defective
25   for the production of Nef or Vpr produced similar amounts
of cell-free virus particles, which were in both cases
considerably less than in the parent virus strain. The
effect of Vpr on virus replication appears to be mediated
by the H(S/F)RIG motifs, as shown in Table 4.

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Table 4

Effect of Deletion of the H(S/F)RIG Motif
on the Replication of Virus PBMC,
as Measured by Cell-Free RT Activity

DAY (P.I)	HIVNL 4.3	VPR MOTIF (-)
3	7440	3878
7	269002	174735
10	219986	165945
15	109533	57493
17	93679	42692

A mutant provirus that was defective in the production of both Nef and Vpr was severely repressed in virus production, and showed delayed replication kinetics (Figure 9a). In unstimulated human primary cells, which closely resemble the *in vivo* cell population, both Nef and Vpr are indispensable for cell-free virus production (Figure 9b). The Vpr⁻ mutant produces smaller amounts of virus, the Nef⁻ mutant exhibits delayed replication kinetics, while the Nef⁻Vpr⁻double mutant shows no virus production. Therefore Nef and Vpr appear to act synergistically.

Example 10   H(S/F)RIG Motifs in Synthetic Peptides
                    Cause Growth Arrest in Yeast

Peptides were dialysed against PBS and added at a final concentration of 2 μM to yeast cells suspended to a density of 10^6 cells/ml in a final volume of 200 μl water. After incubation for 1 h, 5 x 10^4 cells were spread on to solidified YEPD, and the plates were examined for colony growth after 40 hours incubation at 28°C. Peptide concentrations were determined by quantitative amino acid analysis of peptide solutions.

The addition of Peptide 2 or Peptide 3 caused the cells to completely lose colony forming ability, while the

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addition of Peptide 1 had no effect (Figure 10). This
compares with Example 5, in which we found a correlation of
bioactivity, as assessed by osmosensitivity, with the
intracellular presence of H(S/F)RIG motifs.

5   Therefore we further investigated peptides, such
as Peptide 4, which contained only the H(S/F)RIG motifs,
and which also caused some osmosensitivity, as shown in
Figure 11. Peptide 4 also caused complete loss of colony
forming ability. Peptide 5, which is like Peptide 2 but
10   lacks the cysteine, also caused a considerable effect,
suggesting that the cysteine was not essential for the
activity, but that it did increase the activity, possibly
due to a conformational effect.

          We used Peptide 3 to establish a dose response
15   relationship. Treatment of cells with a range of
concentrations of Peptide 3 indicated that the lowest
peptide concentration which induced complete growth arrest
was about 1 μM, as shown in Figure 12. Concentrations down
to 0.05 μM were partially effective, but below this
20   concentration there was no effect.

Example 11   Blockage of the Growth Arrest Effect by
                    Cell Mass
          There was also an effect caused by cell
concentration. At high cell concentrations the
25   effectiveness of the peptides was limited. Figure 13 shows
colony formation after treatment of yeast at a range of
cell densities with 5 μM of Peptide 3. At cell densities
up to 10^5 cells/ml a complete effect can be seen; however,
at concentrations above 10^6 cells/ml no effect was
30   observed. This suggests that about 10^6 molecules of
peptide per cell are required for inhibition of colony
formation. We have also observed that the effect may be
abrogated by the presence of medium; for example the effect
is greatly reduced in the presence of YEPD even at
35   concentrations as low as 1/10 of normal strength.

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Example 12   Effect of Synthetic Peptides on Other
                    Microorganisms

       We have investigated the effects of these
peptides on the growth of several additional
5   microorganisms, and the results are shown in Table 5.

Table 5

Effect of Peptide on Colony Formation of Bacteria, Budding Yeasts and Fission Yeast

Peptide	% "kill" after peptide treatment					
	S. cerevisiae	C. albicans	C. glabrata	K. lactis	Sz. pombe	E. coli
None	0	0	0	0	0	0
1	0	0	0	0	0	0
2	100	100	100	100	100	100
3	100	100	100	100	100	100
4	100	90	99	100	98	100
5	93	90	99	100	78	61

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In *E. coli* and three budding yeasts, *Candida albicans*, *Candida glabrata* and *Kluyveromyces lactis*, and in the fission yeast *Schizosaccharomyces pombe*, the results were similar to those seen with *S. cerevisiae*. An effect
5   was also seen on mammalian cells, in which the peptides inhibit the formation by RC2a cells of a lawn on a culture plate surface.

We treated *E. coli* with Peptides 1, 2 and 3 in the same way as yeast, except that the treated cells were
10   suspended in 2% glucose/50 mN HEPS. The data in Table 5 show that peptides containing the H(S/F)RIG motif affect the viability of *E. coli*. However, we found that when *E. coli* was suspended in PBS and treated with these peptides, no loss of colony-forming ability was observed.
15   It therefore appears that the activity is dependent on the medium used.

Example 13   Effect of Peptides on Yeast Cell
                  Permeability

We examined cells with Fungolight Live/Dead Stain
20   (Molecular Probes), and found that cells remained alive, ie. metabolically active, for several hours after the peptide treatments; however, they had lost colony forming ability. Thus the effect of the peptides seems to be to produce an irreversible growth arrest.
25   We further examined the peptide-treated cells by staining with propidium iodide, a vital stain, followed by flow cytometry. The results, shown in Figure 14, confirm that after 1 h there is a marked effect caused by Peptides 2 and 3. With Peptide 1 or no treatment, 95% of the cells
30   take up little or no propidium iodide. With Peptides 2 and 3, fewer than half of the cells take up propidium iodide. Propidium iodide uptake is usually indicative of cell death or of membrane damage to the yeast. The analysis also shows that there is no significant cell lysis at this time.
35   Kinetic analysis of the effect of Peptide 3, shown in Figure 15, indicates that this effect is

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immediate, with cells taking up propidium iodide within
minutes of the peptide being added.

<u>Example 14</u>   <u>VPR Peptides Kill CD4⁺ Cells</u>

                We have used two CD4⁺ cell lines, RC2a and
5   Jurkat, to represent promonocytic and T lymphocytic cell
lines respectively.

Electroporation of peptides

                10 μg of peptide was added to 1 million CD4⁺
cells which were suspended in 55 μl Baekon buffer (1.5 mM
10   Na_2HPO_4, 0.5 mM KH_2PO_4, 0.27 M Sucrose pH 7.0) and 10 μl of
PBS (phosphate-buffered saline). Electroporation
conditions in the Baekon 2000 Advanced Macromolecule
Transfer System (San Francisco, CA) were 2^{11} pulses, 8 kV,
0.8 sec burst time, 62.5 μsec pulse time, 3 cycles, 85 mm
15   gap between solution and upper electrode. Peptide-
electroporated cells were resuspended in 1 ml RPMI-1640 10%
HIFCS and incubated in a humidified 5% CO_2 incubator at
37°C.

Re-electroporation and pre-treatment of cells

20   Peptide-electroporated cells were harvested at
24 h for re-electroporation of the respective cells with
peptides or without peptide, and the cells were analysed by
flow cytometry after 24 h. One million cells were
pretreated either with 0.5 nM TMB-8 hydrochloride
25   ([8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate], HCl) or
0.5 μM prostaglandin E2 for 30 min before electroporation.
Cells were maintained in the same concentration of the
respective reagents after electroporation.

Preparation of cells for flow cytometry

30   One million cells were harvested and washed once
in PBS at 1600 rpm for 5 min, and the pellet was
resuspended in 200 μl PBS containing 2 μg propidium iodide
in preparation for flow cytometry analysis. Cells were

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analysed for a number of parameters 24 and 48 h after
electroporation, using a Coulter Epics Elite Flow
Cytometer. Forward and side scatters of a 488 nm argon ion
laser were measured. Propidium iodide exclusion was
5   measured by the absence of fluorescence emission at greater
than 600 nm.

Figure 16 shows that Peptide 3 kills RC2a cells
to a significant extent, compared to the mock
electroporated cells. Peptide 2 killed a lower number of
10   cells than did Peptide 3, and the results were comparable
in both cell lines. However, Peptide 1, which lacks the
H(S/F)RIG motif, does not affect these cell lines. The
effect of Peptides 2 and 3 was enhanced by pretreatment
with prostaglandin E2, and hence obviates the need for
15   double electroporation. The effect of the peptide is
modified by pre-treatment with the Ca^{2+}-channel blocker
TMB-8 HCl. The H(S/F)RIG motif also influences the cell
structure as measured by forward and side scatters, as
shown in Figure 17. Peptide 3 has produced a right shift
20   of both side and forward scatters compared to the mock
electroporated and other peptides electroporated cells.
This shows that Peptide 3 induces an increase in both cell
size and cellular granularity.

Example 15   Blockage of Yeast Cell Growth Arrest by
25   TMB-8 and High Ionic Strength

The addition of TMB-8 for 30 min prior to the
addition of the peptides abrogates the effect of Peptide 3,
as shown in Figure 18.

Various concentration of TMB-8 were preincubated
30   with yeast cells for 30 min and then the cells were
incubated in the presence or absence of 5 μM Peptide 3.
Cells were then assayed for colony formation. With no
TMB-8 there were no colonies formed. Low concentrations of
TMB-8 gave protection to about 13% of cells.

35   TMB-8 caused some toxicity at high concentration,
but at lower concentrations no toxicity was observed. At

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these lower levels of TMB-8 Vpr peptides were not totally effective in inhibiting the colony forming ability of yeast cells.

Sodium, calcium, potassium or lithium ions totally abrogated the effect of Vpr peptides, if added 30 mins before or immediately after the addition of Peptide 3. The osmotic support sorbitol also provided partial protection, and total protection was provided by incubation with 0.1 x YEPD. These results are summarised in Table 6. This apparent protection in the presence of salts is due to the inability of yeast cells to bind and internalise the peptides containing the H(S/F)RIG motif. However, in the case of mammalian cells the uptake of these peptides was observed in normal serum-containing medium.

Table 6

Negation of Effect of Peptide 3 on Yeast Colony Formation

Other Treatment	% colony formation with Peptide 3 treatment	
	plus other treatment before or immediately after peptide addition	with other treatment 30 min after peptide addition
0	0	0
0.5 M NaCl	100	3
50 mM NaCl	100	
5 mM NaCl	1	
0.8 M LiCl	80	
60 mM KCl	100	
0.1 M CaCl$_2$	100	
0.1 x YEPD	100	
0.3 M sorbitol	25	

Example 15   Viability of Bacterial Cells Producing Vpr

Cells were treated with the inducer IPTG and aliquots plated on to 2 x YT + Ampicillin plates after appropriate times, and the number of colonies was counted

after overnight incubation of the plates. The data show
that the production of GST and GST-Vpr.BE (encoded by the
BamHI-EcoRI fragment of vpr) does not kill E. coli cells,
but induction slows the growth. The production of GST
5   fused to the full-length Vpr protein leads to similar
effects after three hours induction; however, after 30
hours in inducer there is an actual reduction in the number
of ampicillin resistant cells/ml. This is despite the
culture reaching a typical optical density after overnight
10   incubation, and indicates that the vast majority of cells
in the culture have lost their Amp^r determinant (and no
longer express vpr). Furthermore it implicates the C-
terminal region of Vpr in the cell death. It is also clear
that the uninduced cells have not increased in number,
15   indicating a cytostatic effect in the absence of inducer.
However, the cells grew to 10^8 cells/ml, suggesting that
the toxic effect may be specific to a particular condition,
such as growth in spent medium.

Table 7

Protein produced	[Amp^r cells/ml] and time after induction				
				30 hours	
		-inducer	+inducer	-inducer	+inducer
GST	1.1×10^8	4×10^8	2.2×10^8	4.8×10^9	4.4×10^9
GST-Vpr.BE	6.8×10^7	2×10^8	4×10^7	4.8×10^9	2.2×10^9
GST-Vpr	1.3×10^8	4×10^8	1.4×10^8	1×10^8	$<2 \times 10^6$

25   Example 16   Interaction of Fluorescence-Labelled
                     Peptides with Cells
           Figure 19 shows the association of FITC
(fluorescein isothiocyanate)-labelled peptides with $CD4^+$
human cells measured by flow cytometry following (A)
30   electroporation, and (B) extracellular addition without
electroporation.

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Figure 20 shows the association of FITC-labelled peptides without electroporation in *S. cerevisiae* yeast cells measured by flow cytometry. Peptides 2-4 exhibit high degrees of association with yeast cells, while there

5   is a considerably lesser association of Peptide 5. Peptide 1, which lacks the H(S/F)RIG motif, exhibits over one hundred-fold less association with cells than Peptide 2 and 3. By light microscopy we have observed that the FITC-labelled Peptide 3 efficiently targets into yeast and

10   mammalian cells, as shown in Figure 21. Peptides 2 and 4 also behave similarly. These data indicate that the H(S/F)RIG motif are sufficient for intracellular targetting and they, or related derivatives that could also be a subset of the sequence, will be useful carriers for the

15   delivery of agents into cells for treatment of diseases.
       Figure 21 shows the internalised FITC-labelled peptide 3 in human CD4$^+$ cells (A) and in yeast cells (B). The FITC-labelled human cells include some cells that are still intact and others undergoing lysis.

20   Example 17   Genetic Interaction Between Vpr and Sac1 p
                           and Actin
       Yeast *act1* and *sac1* mutants, DBY1195 and DBY1715 respectively, were transformed with pYEULCBX and pYEULCBX.Vpr. Transformants were then induced on plates

25   containing 0.5 mM copper sulfate to assay for the effects of the Vpr. DY150 [pYEULCBX] and DY150 [pYEULCBX.Vpr] transformants have been described previously. An example of these results for the *sac1-vpr* interaction is shown in Figure 22.

30   We have found that Vpr shows structural homology to the yeast protein Sac1p. The precise function of Sac1p in assembly of the actin cytoskeleton of yeast cells is still under investigation; however, *sac1* mutants display profound cytoskeletal defects and growth arrest at low

35   temperature (Cleves *et al*, 1989; Novick *et al*, 1989; Whitters *et al*, 1993). Production of Vpr in yeast possibly

causes similar effects to *sac1* mutants, due to sequence and functional similarity between Sac1p and Vpr; the production of Vpr could compete with normal Sac1p function and lead to cytoskeletal defects, including gross cell size and
5 ultimate growth arrest. Indeed, in the many studies of yeast with cytoskeletal defects, mother cells are abnormally large and daughter cells are abnormally small (see for example Liu and Bretscher, 1992). We have found that time-lapse analysis of newly-induced cells producing
10 Vpr shows the same phenomenon. Osmosensitivity also indicated possible cytoskeletal defects induced by Vpr. Large cells producing Vpr were isolated by flow cytometry and plated onto media containing high osmotic strength and normal media. Only 50% of the cells capable of growth on
15 normal medium could grow on high osmotic strength medium, indicating structural defects in those cells.

Vpr appears to produce cytoskeletal defects in mammalian cells as well as in yeast cells. Work by Levy and colleagues showed that in a rhabdomyosarcoma cell line
20 Vpr produced cell replication arrest and gross cell enlargement (Levy *et al*, 1993). Furthermore, in a CD4⁺ T-lymphoblastoid cell line it was shown that HIV-1 caused ultrastructural changes, including membrane disruption, "ballooning" and vacuolisation of the endoplasmic
25 reticulum, during the first hour of infection (Fermin and Garry, 1992). These data are consistent with cytoskeletal defects, and investigation of the cytoskeleton in those cells would be of interest.

What is the role of Vpr in the HIV-1 life cycle,
30 and is induced growth arrest relevant to this? For some time there has been a dilemma regarding the distinction between HIV-1 and other retroviruses: retroviruses usually require cell proliferation for infection, while HIV-1 infects non-proliferating cells such as terminally-
35 differentiated macrophages. Lewis *et al* (1993) showed that CD4⁺ cell lines can be productively infected with HIV-1 when they are arrested in G2 growth phase. Non-

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proliferation of host cells could therefore be an initial
requirement for a productive infection of all or some cell
types. The function of Vpr may be to bring about growth
arrest so that a process like integration may occur. If
5   this were so, it would account for Vpr (and Vpx
counterparts) being virion-associated, so that early events
can be initiated. Antibodies to Vpr have been detected in
only 17% of AIDS patients, but are found in 47% of
asymptomatic individuals (Wong-Staal et al, 1987),
10   suggesting that the Vpr is present early in infection, and
therefore that it is probably essential only at that time.
It also follows then that inhibitors of Vpr should prevent
infection or slow extracellular spread of the virus.

In this study we have shown that portions of Vpr
15   containing the sequence HFRIGCRHSRIG or even RHSRIG cause
cell damage and irreversible growth arrest when added to
yeast cells. This effect is related to cell number and
peptide concentration, and it appears that a minimum of
10^6 molecules of peptide per cell is required to observe
20   the effect. We have also shown that the same sequence was
involved in causing osmosensitivity and structural defects
when peptides containing this sequence were electroporated
into cells. Only intracellular effects were examined after
electroporation, since YEPD in the electroporation medium
25   abrogated the extracellular effect.

Peptides containing H(S/F)RIG motifs appear to
cause cell damage resulting in increased permeability and
cell lysis in both mammalian and yeast cells. We have
observed that Vpr peptides containing the H(S/F)RIG motif
30   are cell associated; fluorescence microscopy using FITC-
labelled peptides indicates that they are internalised
within 1 h. We have ascertained that the H(S/F)RIG motif
causes active uptake of the peptide; uptake of FITC-
labelled Peptide 1 appeared to be one hundred-fold lower
35   than that of peptides containing the H(S/F)RIG motif,
suggesting that the motif does promote uptake. In Example
7, peptide uptake was artificially obtained by

electroporation, and the outcome studied was
osmosensitivity; the electroporation technique itself led
to variable degrees of cell death or loss of colony-forming
ability. However, among the numerous cells that formed
5   colonies there was a high degree of osmotic sensitivity.
The effects observed in Examples 10 to 15 are quite
different, with total loss of colony forming ability.
These differences may be related to the localisation of the
peptide within the cell. Osmosensitivity, rather than loss
10   of colony forming ability, was also observed with the
expression of the vpr gene in yeast in a more life-like
situation, as described in Example 2.

What AIDS phenomenon then can be correlated with
our results? Recent studies by Levy et al (1994) show that
15   Vpr does exist in the serum, suggesting that it is released
from infected cells, and indicating that in designing
putative antagonists of the protein it is relevant to
consider the extracellular effects of Vpr, which may be
responsible for the killing by HIV of uninfected host
20   cells, as well as its intracellular effects.

Using biologically active fragments of Vpr, we
have shown that parts of Vpr, and presumably the entire Vpr
protein, irreversibly affect colony-forming ability via the
action of the H(S/F)RIG motifs within Vpr. The mode of
25   action of this effect may be related to the Ca^{2+} ion
channel, since the Ca^{2+} ion channel blocker TMB-8 abrogates
the effect, as shown in Examples 14 and 15.

References cited herein are listed on the
following pages, and are incorporated herein by this
30   reference.

It will be apparent to the person skilled in the
art that while the invention has been described in some
detail for the purposes of clarity and understanding,
various modifications and alterations to the embodiments
35   and methods described herein may be made without departing
from the scope of the inventive concept disclosed in this
specification.

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CLAIMS

1.   An antagonist of the Vpr protein of human
immunodeficiency virus (HIV), or of a biologically active
fragment or analogue thereof, comprising at least one amino
acid sequence motif selected from HFRIG and HSRIG, said
antagonist having the ability to inhibit one or more
activities mediated by Vpr, selected from the group
consisting of growth arrest, cell replication arrest,
cytotoxicity, cytoskeletal disruption, and effects on the
endoplasmic reticulum.

2.   An antagonist according to Claim 1, wherein the
Vpr protein further comprises at least one sequence
selected from the group consisting of HSRIS, HFRAG, HIRAG,
HLRAG, RSRKG, RSRIS and RSRIG.

3.   An antagonist according to Claim 1 or Claim 2,
wherein the Vpr protein is a fragment comprising at least
the C-terminal 21 amino acids of the Vpr sequence.

4.   An antagonist according to Claim 3, wherein the
Vpr protein is a fragment comprising at least the
C-terminal 33 amino acids of the Vpr sequence.

5.   An antagonist according to any one of Claims 1 to
4, selected from the group consisting of an antibody, an
anti-sense RNA, and a triple-stranded DNA.

6.   An antagonist according to Claim 5, which is an
antibody.

7.   An antagonist according to Claim 6, which is a
monoclonal antibody.

8.   An antagonist according to Claim 5, which is an
anti-sense RNA.

9.   An antagonist according to Claim 5, which is a
triple-stranded DNA.

10.   A pharmaceutical composition comprising as active
component an antagonist according to any one of Claims 1 to
9, together with a pharmaceutically-acceptable carrier.

11.   A method of screening compounds suspected of
being useful as antagonists of Vpr protein, or of a
biologically active fragment or analogue thereof,

comprising the step of measuring the effectiveness of a
test compound in inhibiting the activity of Vpr in an assay
of a biological activity selected from the group consisting
of growth arrest, cell replication arrest, cytotoxicity,
cytoskeletal disruption, and effects on the endoplasmic
reticulum.

12.   A vaccine for prevention of HIV infection or for
alleviation of the effects of HIV infection, comprising
human immunodeficiency virus-1 or human immunodeficiency
virus-2 from which the portion of the HIV genome encoding
at least the C-terminal 21 amino acids of the Vpr sequence
has been deleted, together with a pharmaceutically-
acceptable carrier.

13.   A vaccine according to Claim 12, wherein the
portion of said genome encoding at least the C-terminal 33
amino acids of the Vpr sequence has been deleted.

14.   A vaccine according to Claim 12 or Claim 13,
wherein both a portion of the genome encoding the C-
terminal region of the Vpr sequence and the portion of the
HIV genome encoding the N-terminal of the Nef gene have
been deleted.

15.   A vpr gene in which the region encoding C-
terminal portions of the Vpr protein has been replaced by
an inhibitory antisense sequence or by a sequence which
encodes an inhibitory peptide.

16.   A method of treatment of HIV infection,
comprising the step of administering to a subject in need
of such treatment an effective amount of an antagonist of
Vpr protein, or of a biologically active fragment or
analogue thereof, as defined in any one of Claims 1 to 9,
thereby to prevent HIV infection, to prevent progression of
HIV infection to symptomatic AIDS, or to alleviate the
symptoms of AIDS.

17.   A method of treatment of a disease mediated by
cell proliferation, comprising the step of administration
to a mammal in need of such treatment of an effective
amount of Vpr protein, or of a biologically active fragment

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or analogue thereof comprising at least one amino acid
sequence motif selected from HFRIG and HSRIG, thereby to
inhibit proliferation of cells mediating the said disease.

18.   A method according to Claim 17, wherein the
disease mediated by cell proliferation is a cancer, a
leukaemia, or psoriasis.

19.   A method according to Claim 17 or Claim 18,
wherein the Vpr protein or fragment or analogue thereof is
used in conjunction with an enhancer of Ca^{2+}-channel
transport.

20.   A method of treatment of a disease caused by a
pathogen, comprising the step of administering to a mammal
in need of such treatment of an effective amount of Vpr
protein, or of a biologically active fragment or analogue
thereof comprising at least one amino acid sequence motif
selected from HFRIG and HSRIG.

21.   A method according to Claim 20, wherein the
disease is caused by a bacterium, a parasite, a yeast or a
fungus.

22.   An agent for delivery of a pharmaceutically
active substance to a cell membrane or to the interior of a
cell, comprising a Vpr peptide or a biologically active
fragment or analogue thereof comprising at least one amino
acid sequence motif selected from HFRIG and HSRIG, said
peptide, fragment or analogue being linked to said
pharmaceutically active substance.

23.   A method of delivery of a pharmaceutically active
substance to a cell membrane or to the interior of a cell,
comprising the step of contacting said cell with an agent
as defined in Claim 23.

24.   Use of Vpr protein, or of a biologically active
fragment or analogue thereof comprising at least one amino
acid sequence motif selected from HFRIG and HSRIG in the
treatment of a disease mediated by cellular proliferation.

25.   Use of Vpr protein, or of a biologically active
fragment or analogue thereof comprising at least one amino
acid sequence motif selected from HFRIG and HSRIG in the

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treatment of a disease caused by a pathogen.

26.   An antagonist of the Vpx protein of human immunodeficiency virus-2 (HIV-2) or of a biologically active fragment or analogue thereof, comprising at least one amino acid sequence motif selected from the group consisting of HCKKG, CLGEG, CLGEE, CLGGE, CLGRG, HVRKG, HYTKG, HFKRG, HFKKG, HAKRD, CLQEG, CLGGG, CRGEG, CWGED, HFRCG and RRQPF, said antagonist having the ability to inhibit one or more activities mediated by Vpx, selected from the group consisting of group arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption and effects on the endoplasmic reticulum.
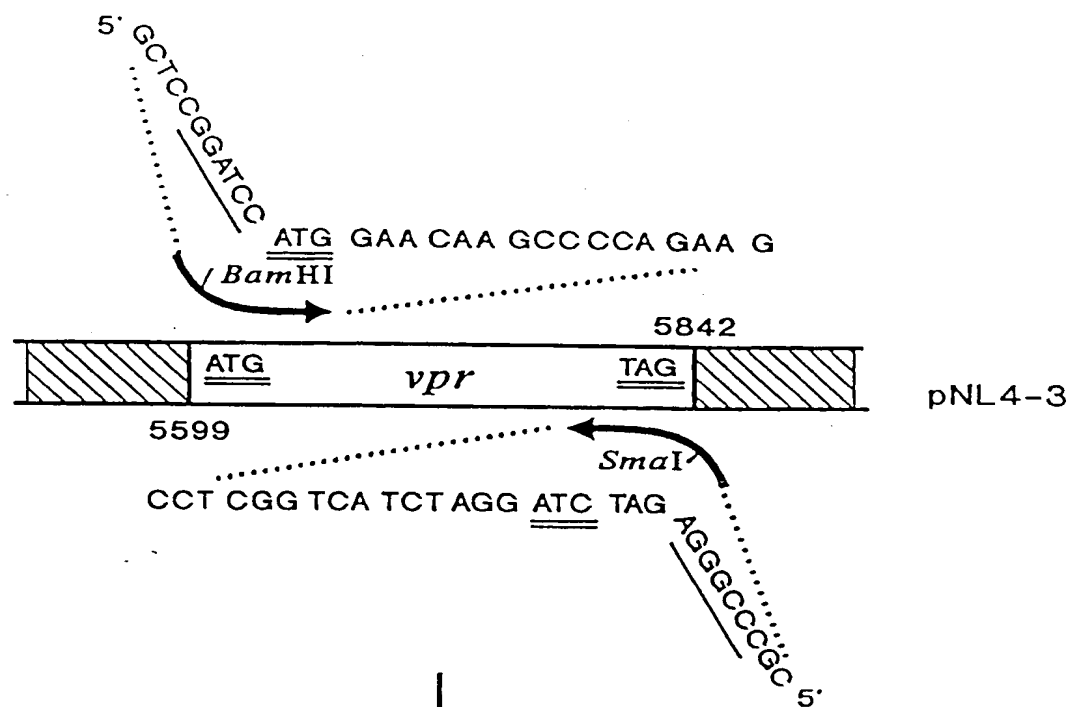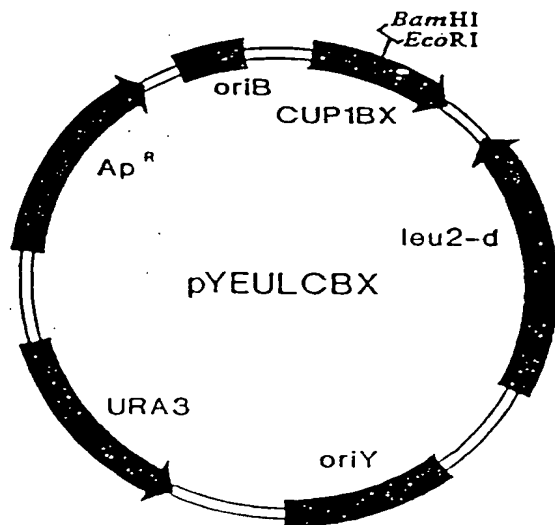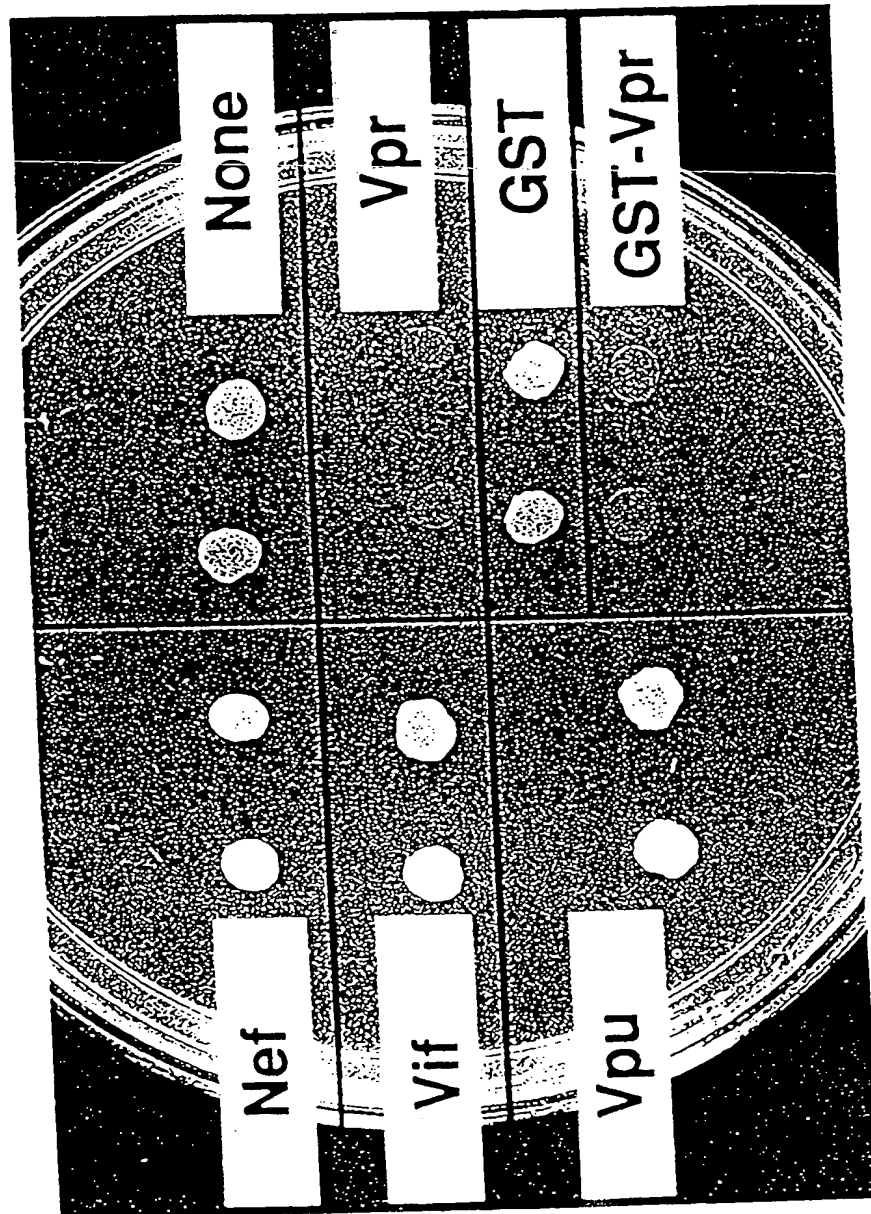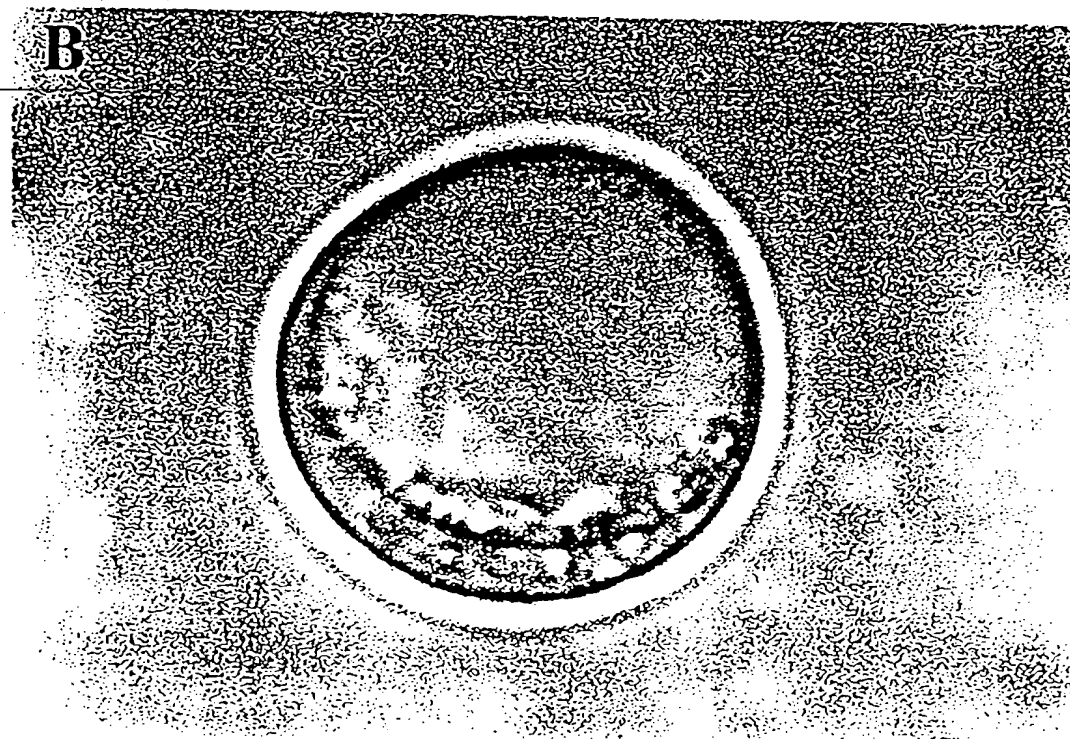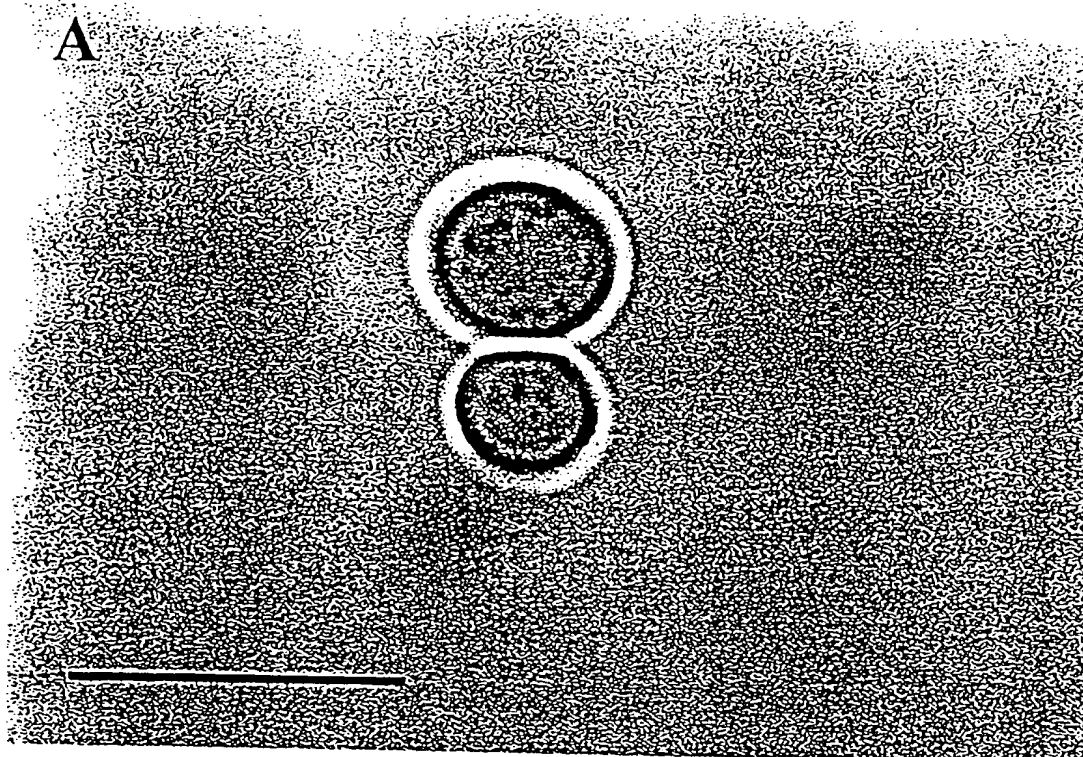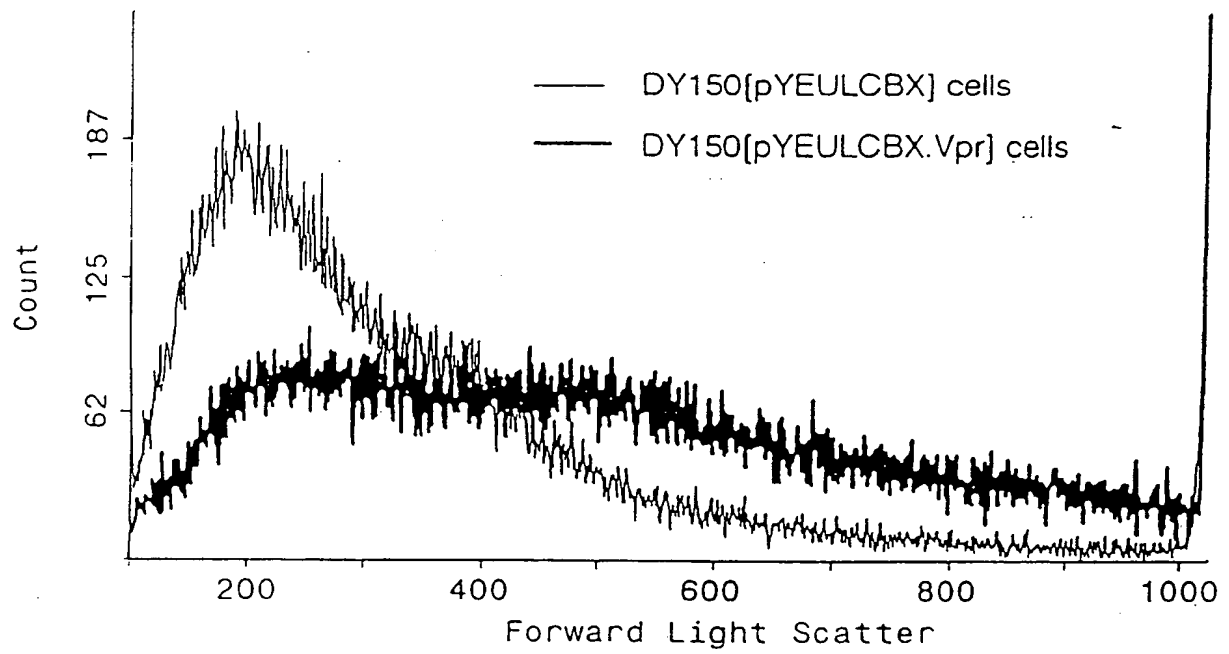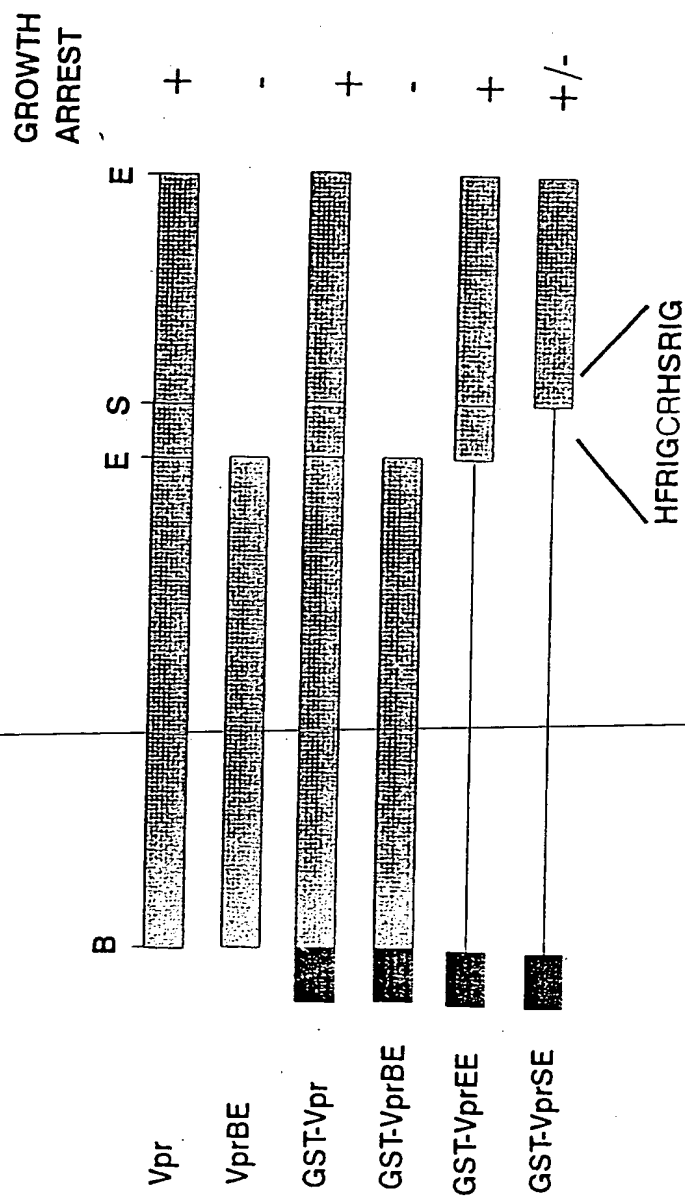
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Alignment of Vpr-relatives

A.

```
HIV-1 Vpr     MEQ-APEDQG-PQREPYNEWTLELLEELKSEAVRHF-PRIWLHNLGQ--HIY   47
SIVmac Vpr    MEER-PPENEG-PQREPWDEWVVEVLEELKEEALKHFDPR-LLTALGN--HIY   48
HIV-2 Vpr     MAEAPTE-LPPVDGTL--REPGDEWIIEILREIKEEALKHFDPR-LLIALGK--YIY   51
HIV-2 Vpx     MTDPRETVPPGNSGEETIGEAFAWLNRTVEAINREAVNHL-PRELIFQVWQRSWRY   55
SIVmac Vpx    MSDPRERIPPGNSGEETIGEAFEWLNRTVEEINREAVNHL-PRELIFQVWQRSWEY   55

HIV-1 Vpr     --ETYG-DTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGA--SRS   96
SIVmac Vpr    --NRHG-DTLEGAGELIRILQRALFMHFRGGCIHSRIGQPGGGNPLSAIPPSRSML  101
HIV-2 Vpr     --TRHG-DTLEGARELIKVLQRALFTHFRAGCGHSRIGQTRGGN-LSAIPTPRNMQ  103
HIV-2 Vpx     WHDEQGMSESYTKYRYLCIIQKAVYMHVRKGCTCLGRGHGPGGWRPGPPPPPPGLV  111
SIVmac Vpx    WHDEQGMSQSYVKYRYLCLMQKALFMHCKKGCRCLGEGHGAGGWRPGPPPPPPGLA  111
```

B.

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Sac1p     ...SLQRNEKVGPAASWKTADERFFWNHYLTEDLRNFA-HQDPRI-DSFIGQPVIY  126
HIV-1 Vpr    MEQAPEDQGP--QREPYNE---WTLELLEELKSEAVRHFPRIWLHNLGQ--HIY   47

Sac1p     -GYAKTVDAVLNATPIVLGLITRRSIFRAGTRYFRRGVDKDGNVGNFNETE...   176
HIV-1 Vpr    ETYGDTWAGVRAIIRILQQLL--FIHFRIGCRHSRIGVTRQRRARNGASRS       96
```

FIGURE 6
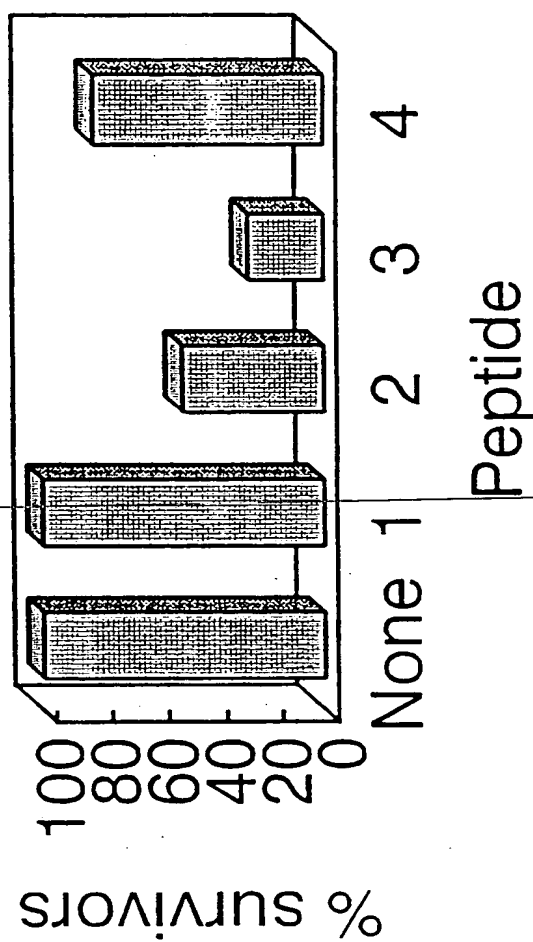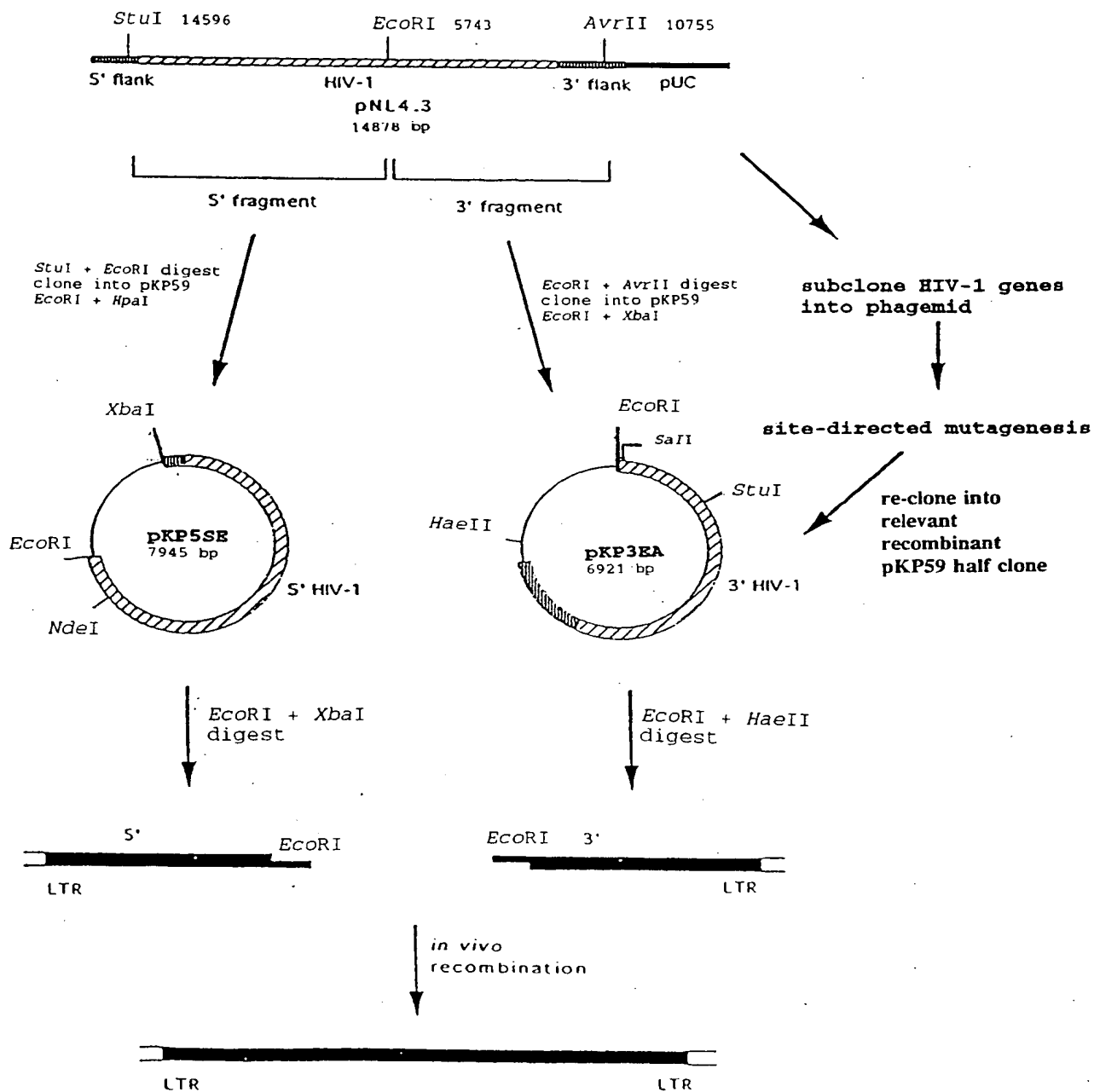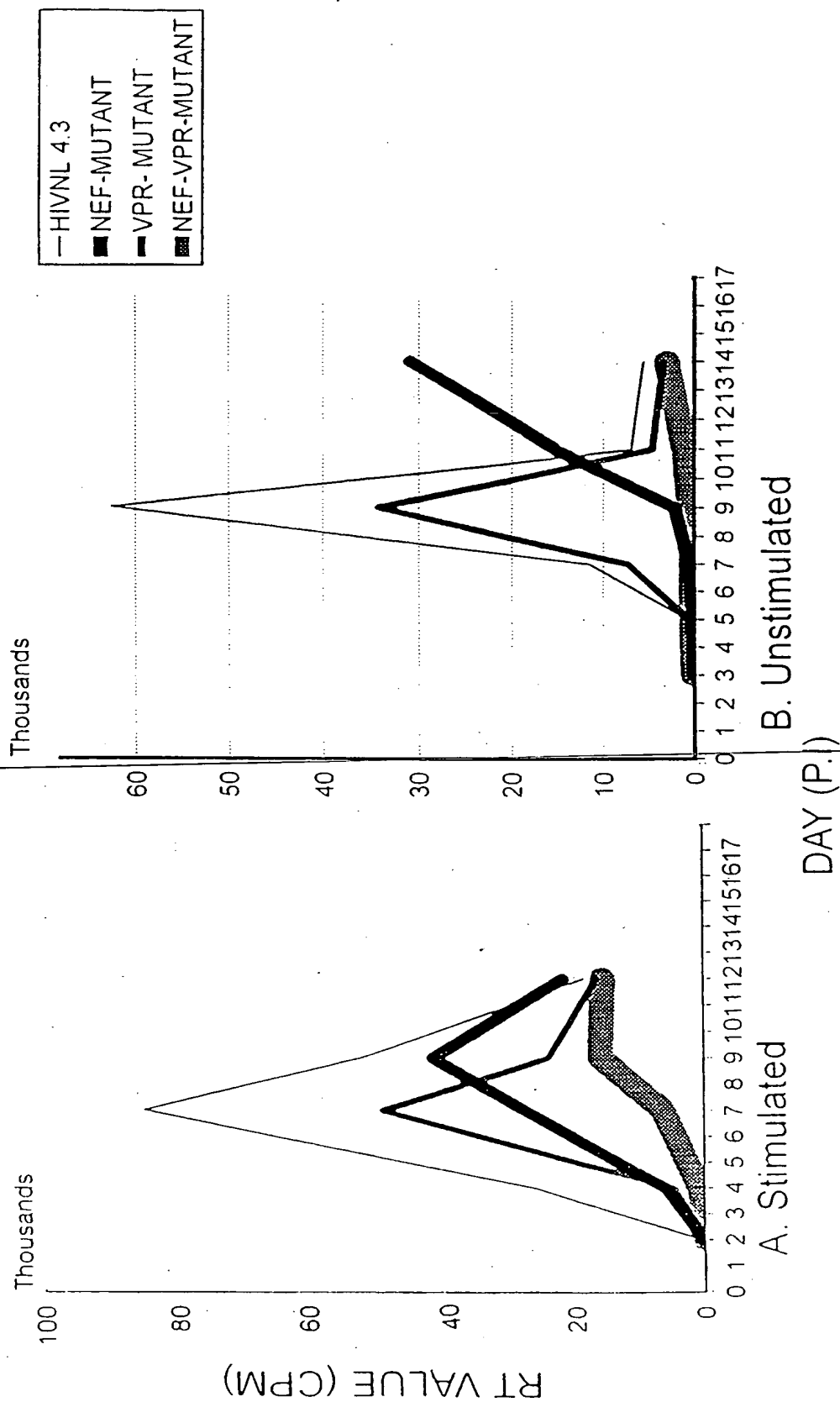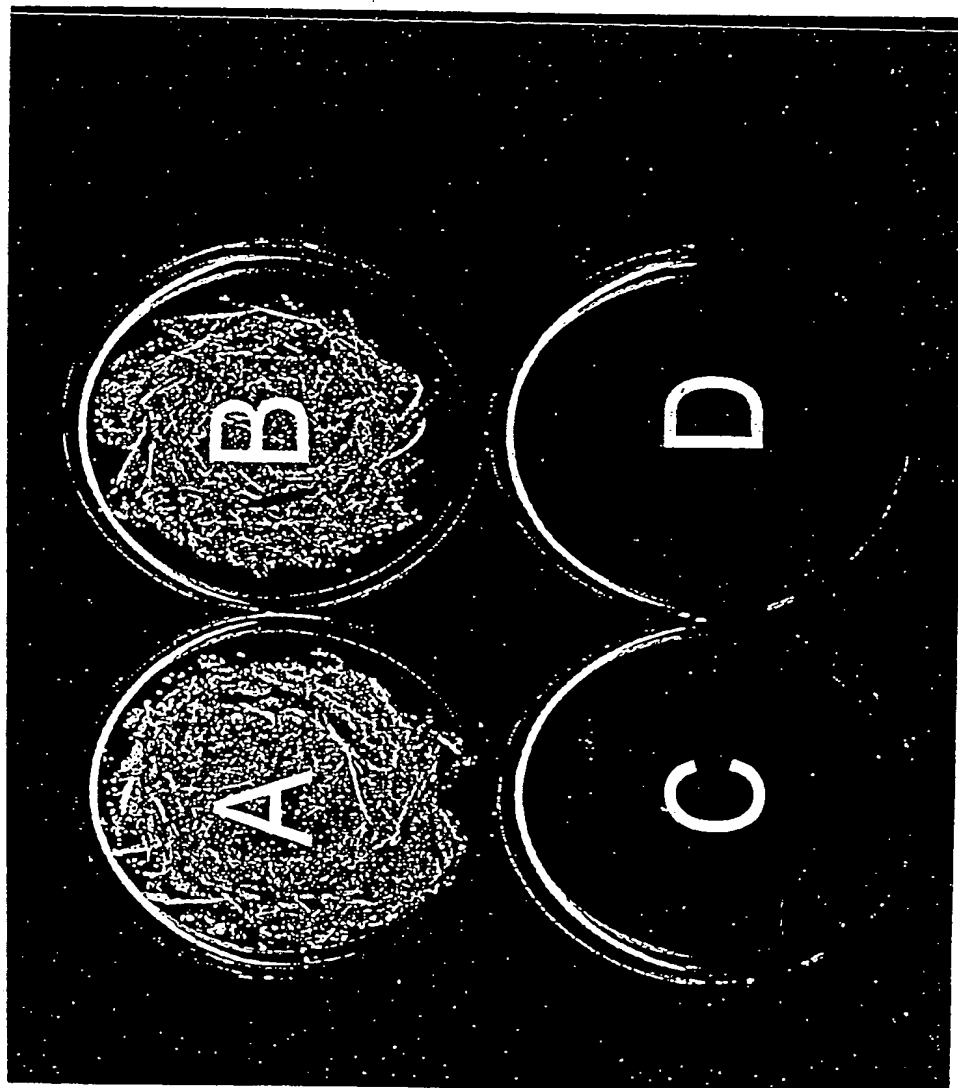
FIGURE 7

FIGURE 8
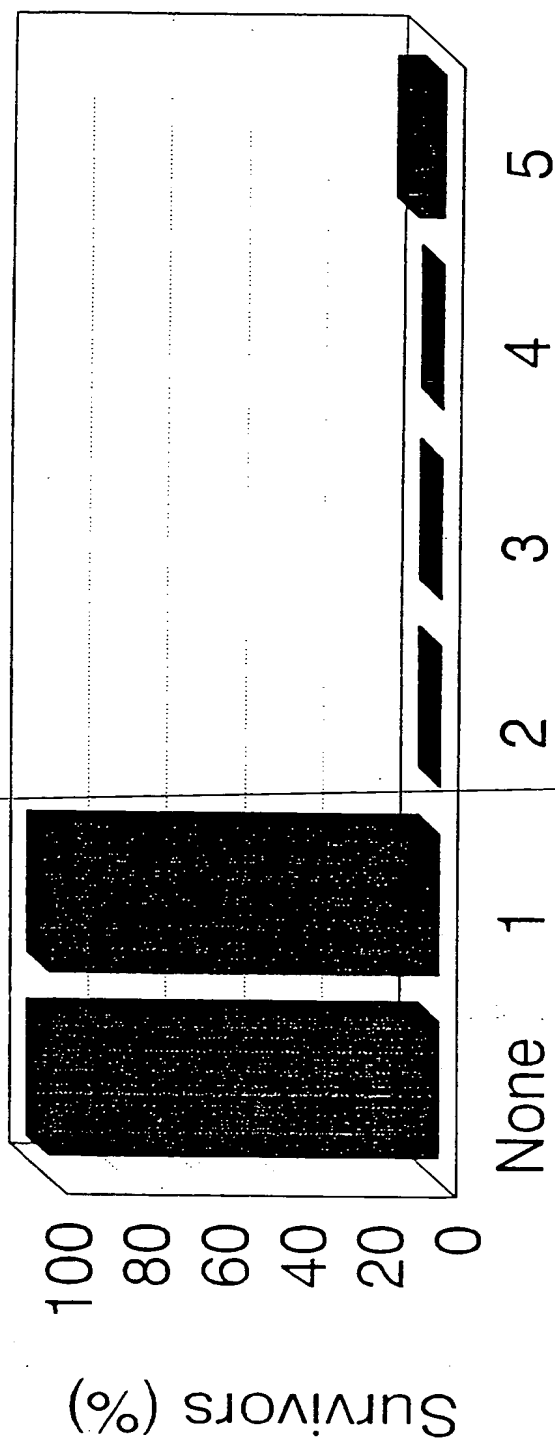
FIGURE 9
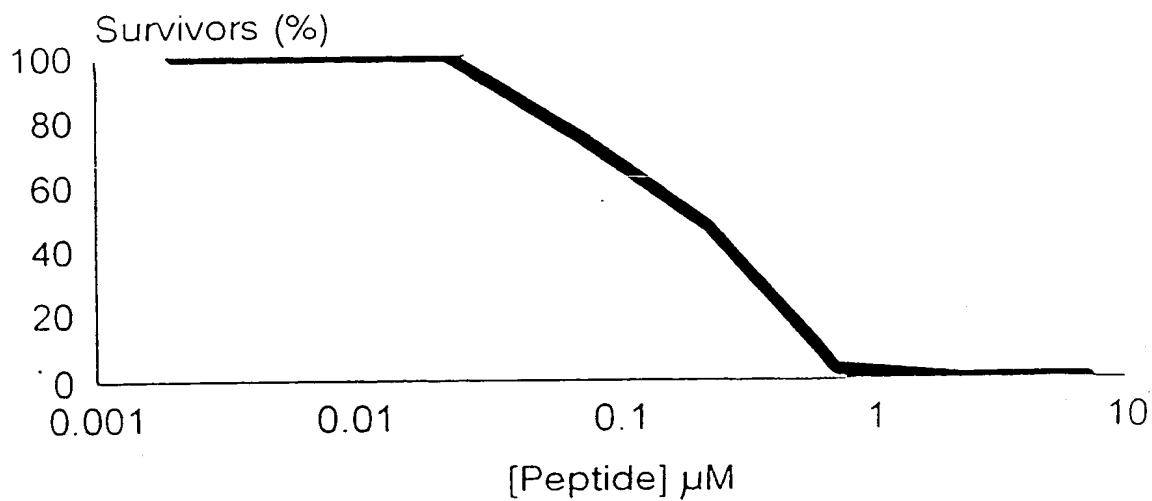
FIGURE 10

FIGURE 11

FIGURE 12

FIGURE 13

FIGURE 14

FIGURE 15

15/21

FIGURE 16

FS — FORWARD SCATTER
SSC — SIDE SCATTER
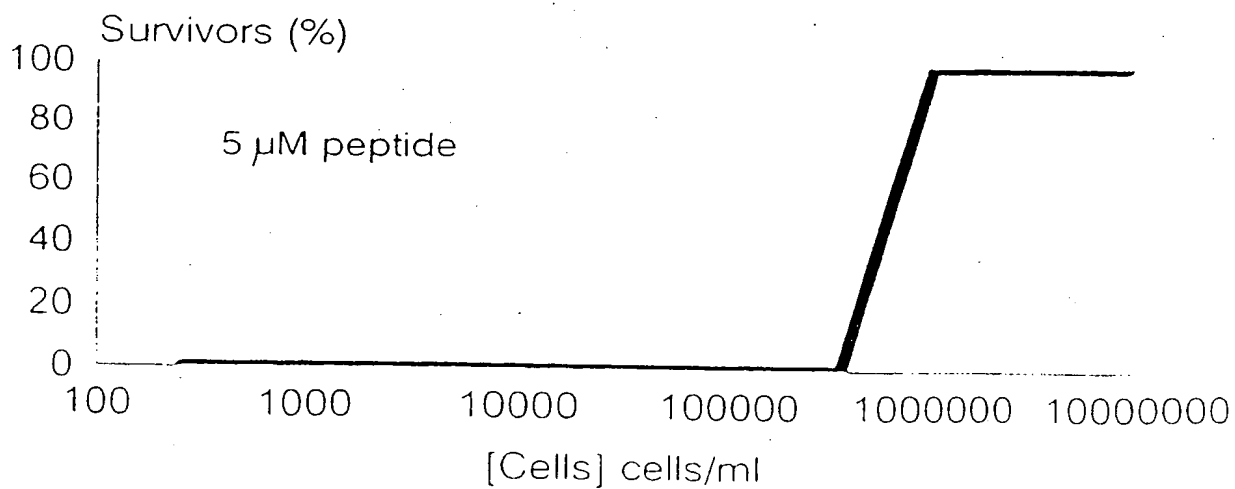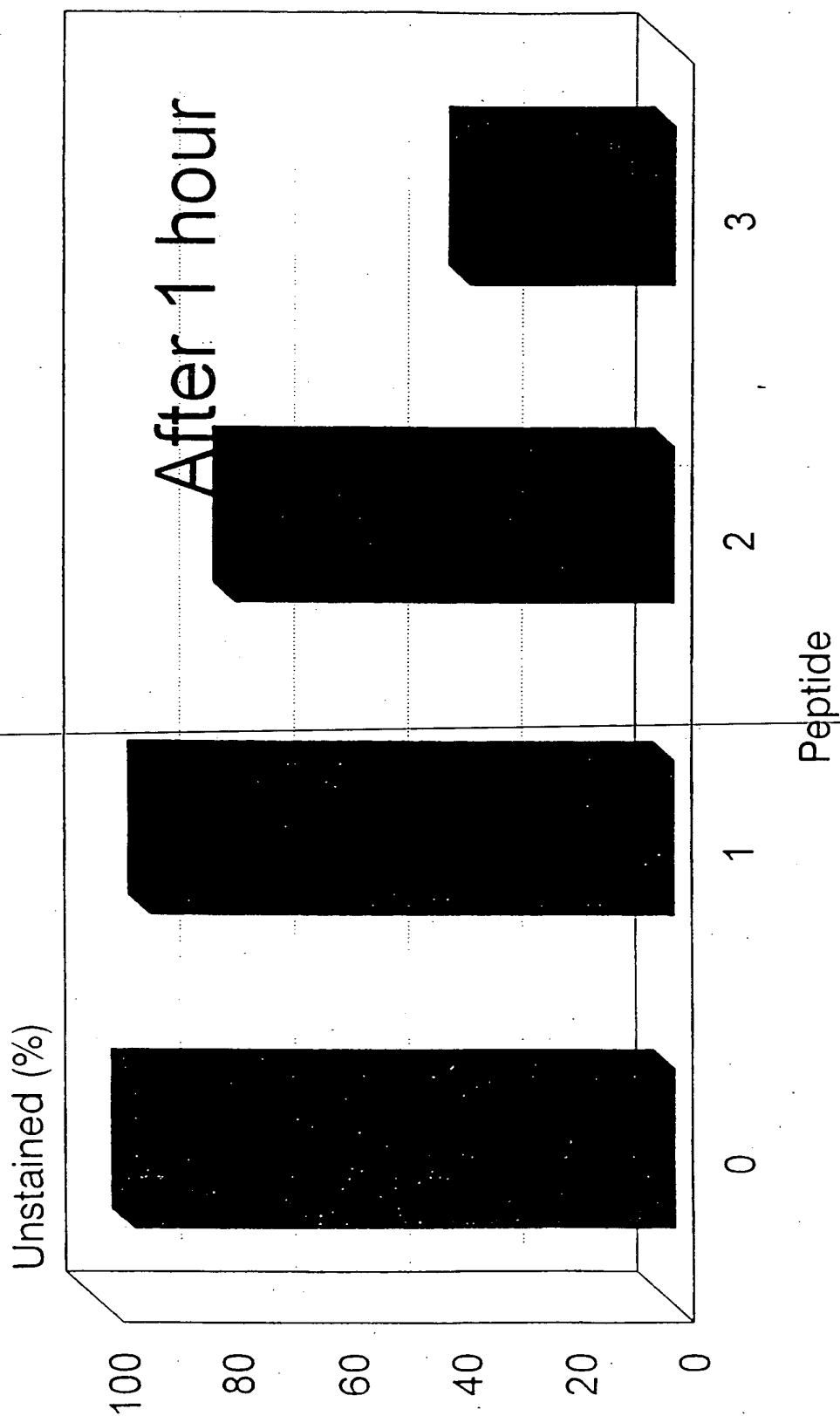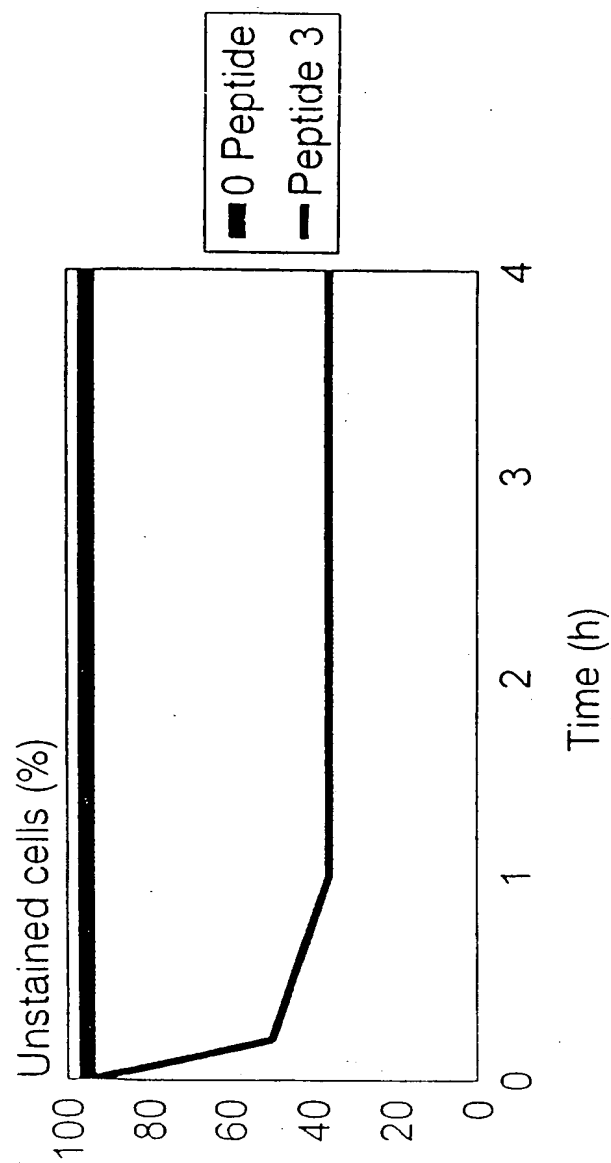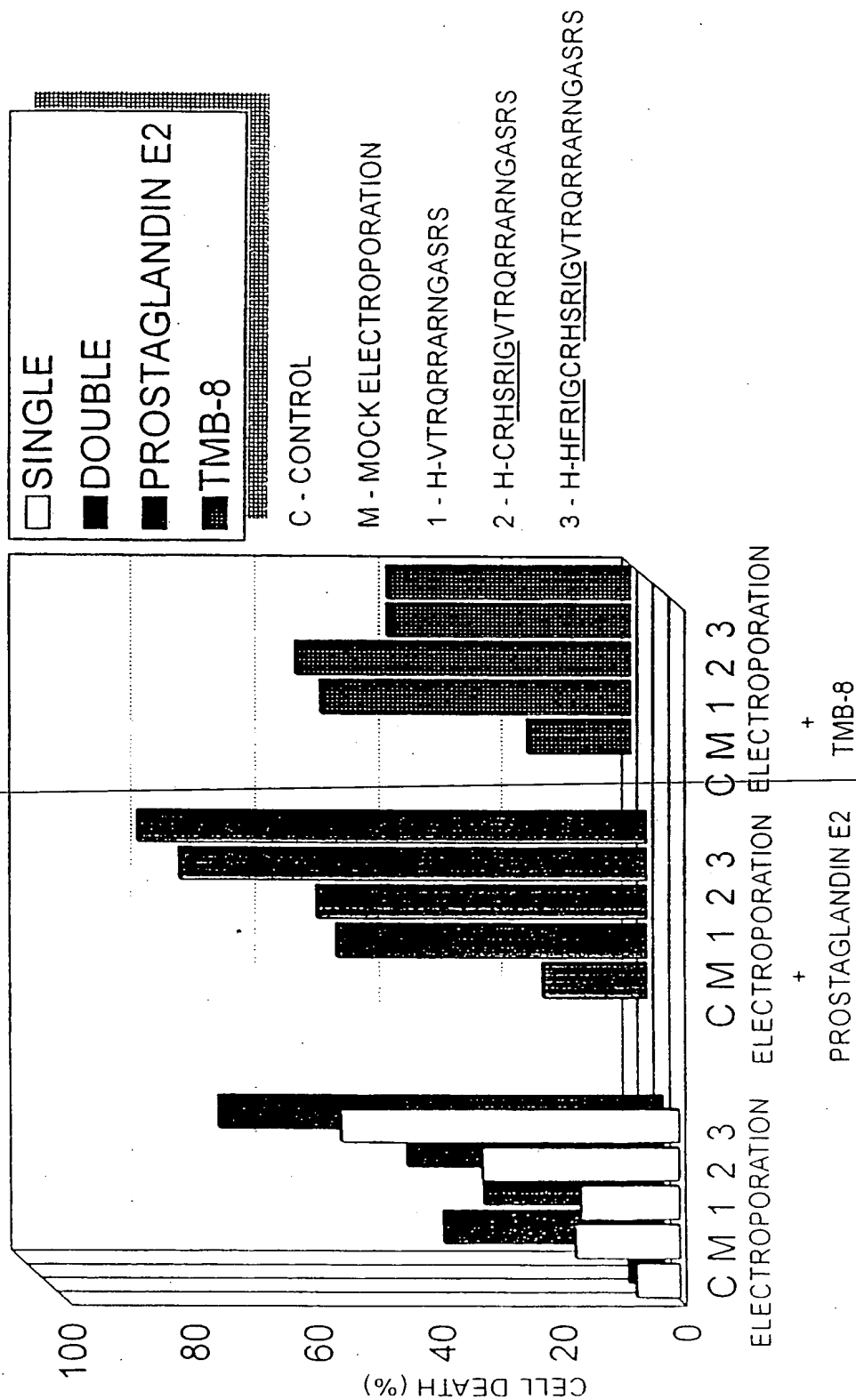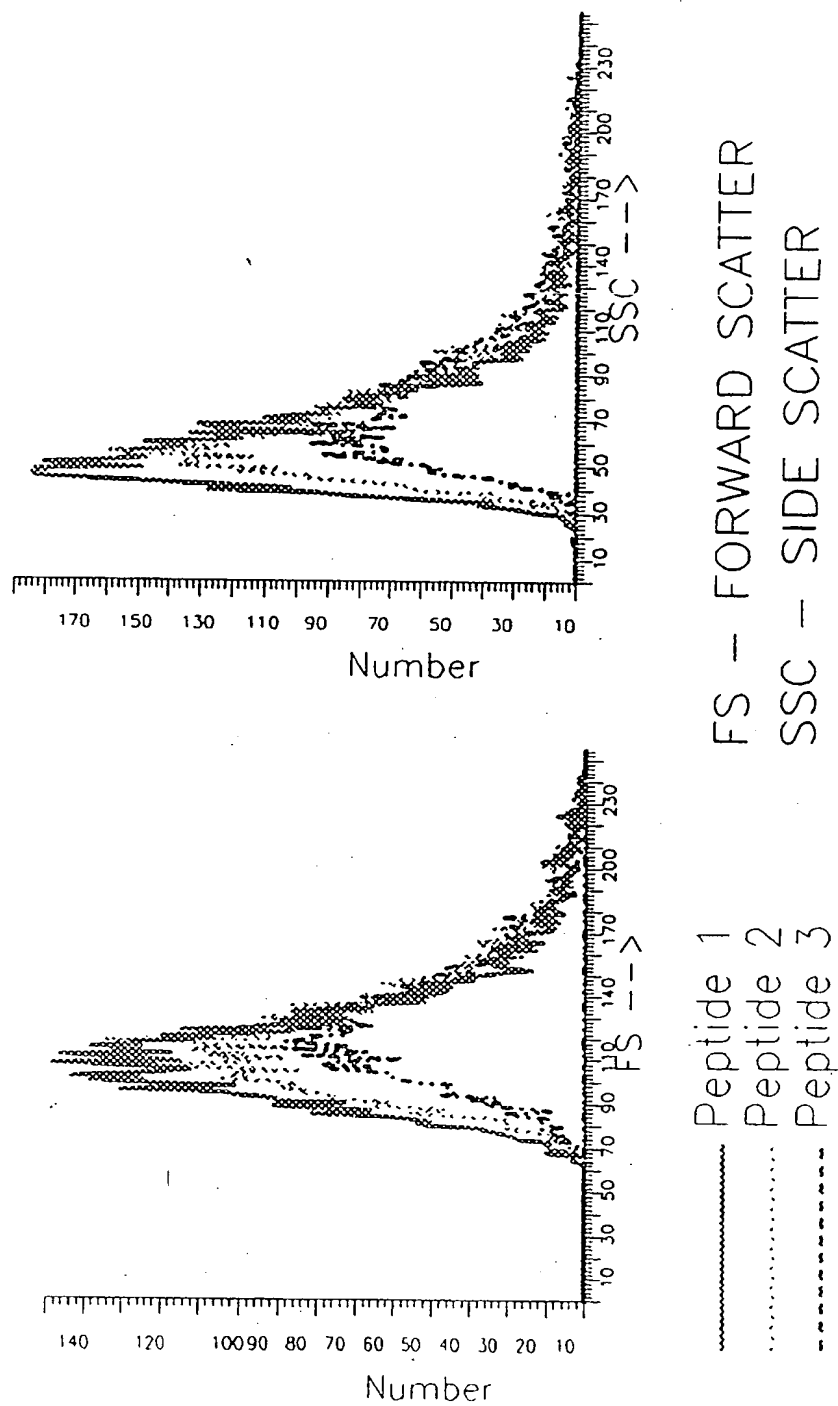
Peptide 1
Peptide 2
Peptide 3
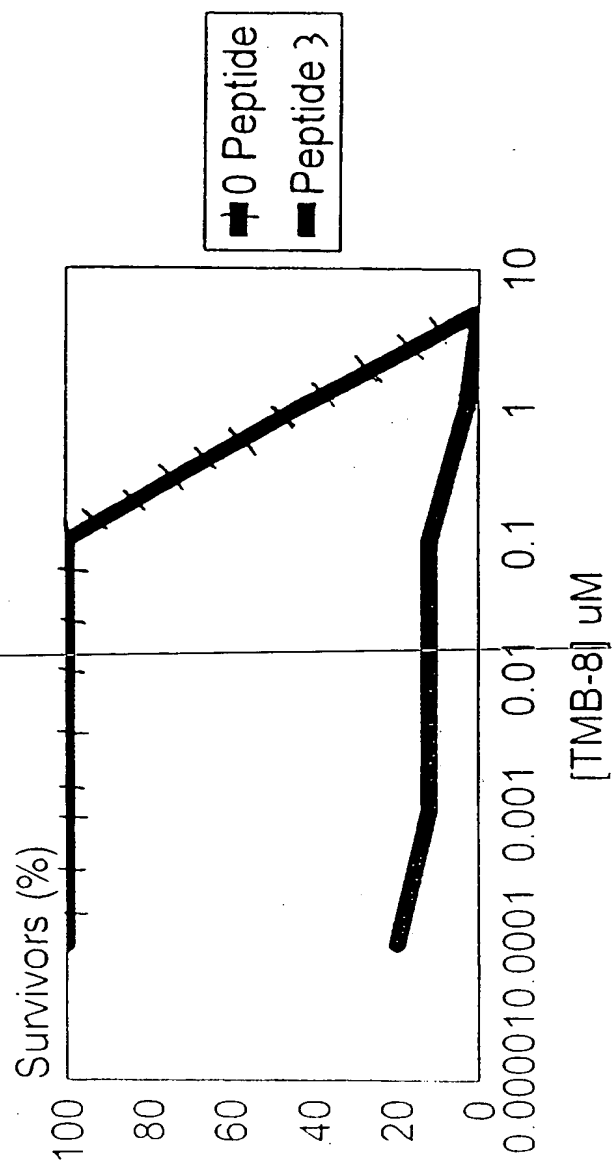
FIGURE 17
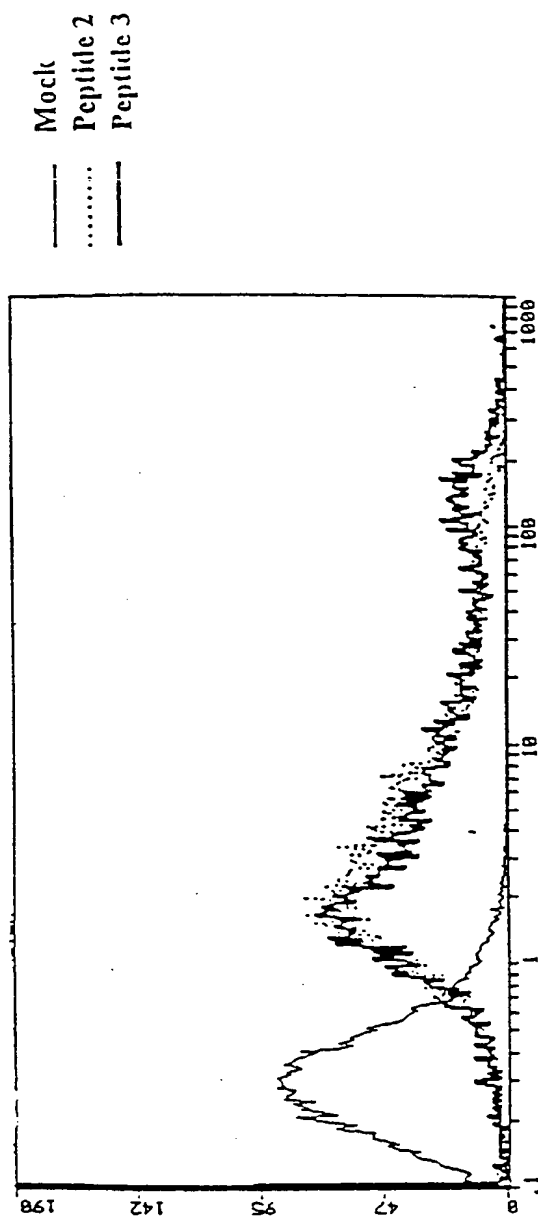
FIGURE 18

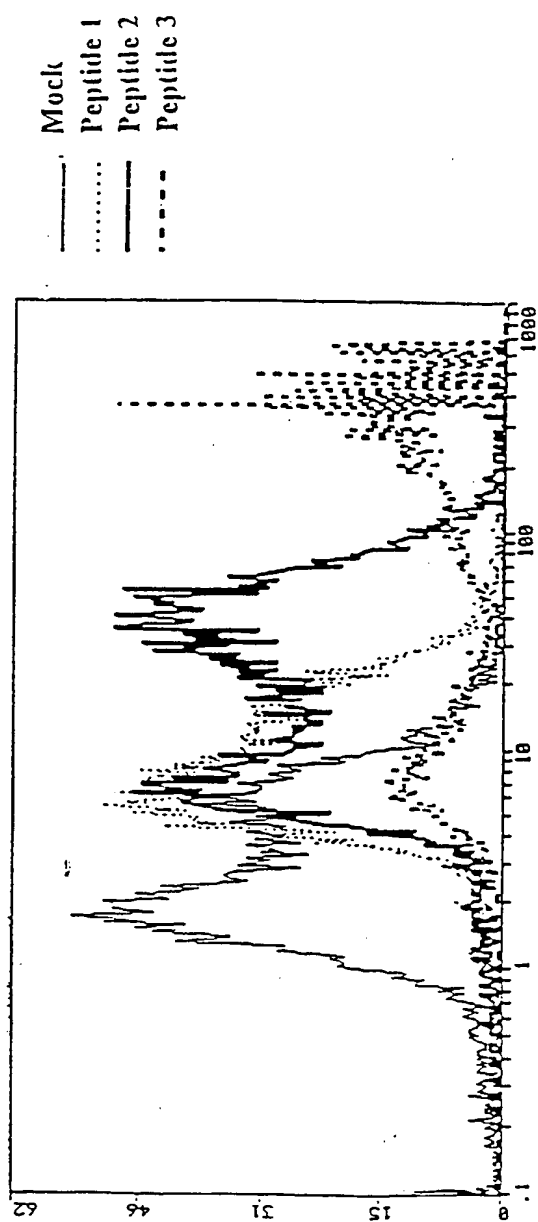A. Uptake of Flourescein labelled peptides with Electroporation

FIGURE 19
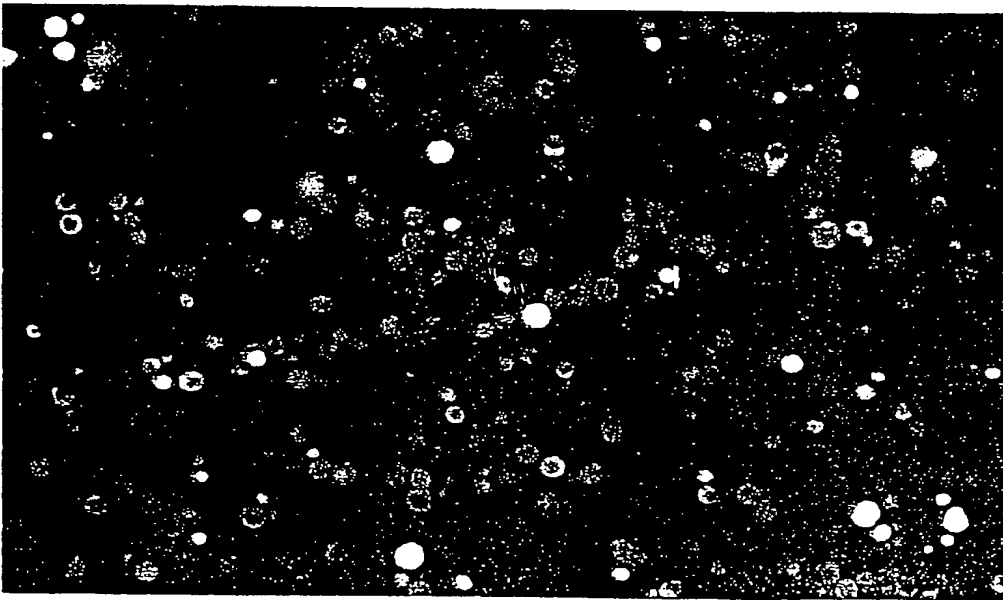
FIGURE 20

FIGURE 21
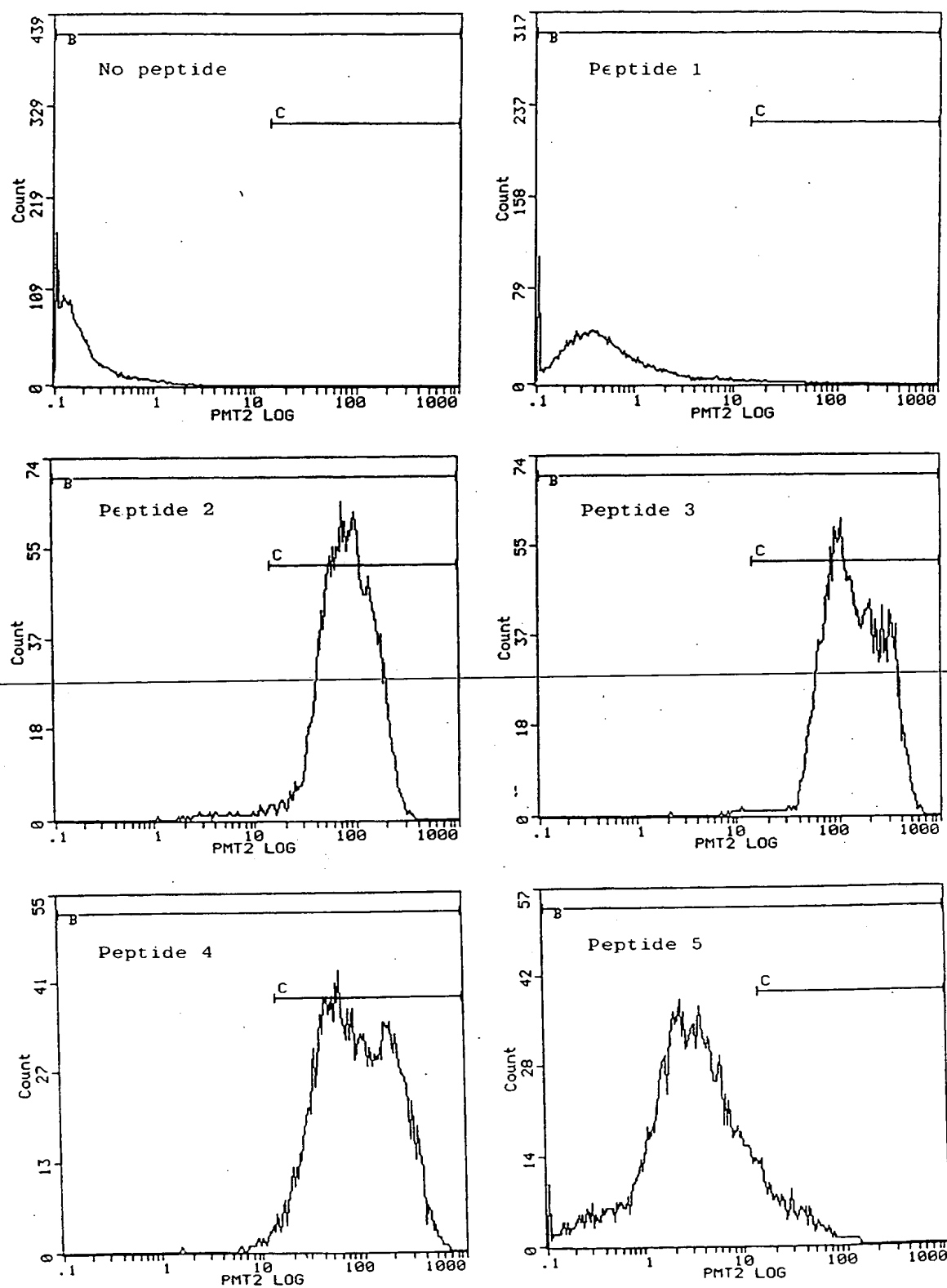
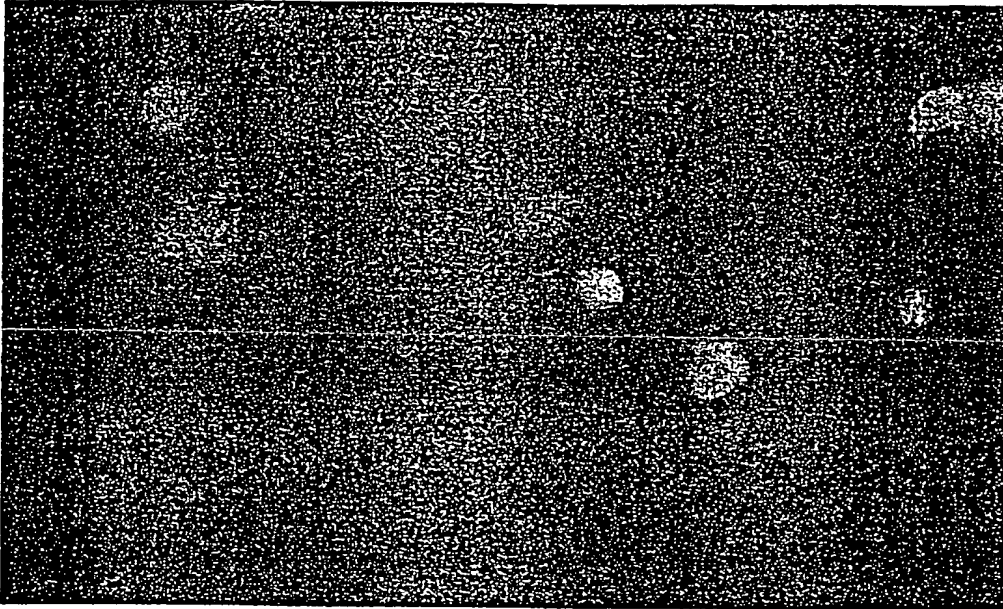| Strain	pYEULCBX		pYEULCBX.Vpr	
	0 Cu	0.5 mM Cu	0 Cu	0.5 mM Cu
Wild-type (DY150)	++++	++++	++	-
act1 (DBY1195)	++++	++++	++++	+++
sac1 (DBY 1715)	++	++	++	++

Vpr interacts with Sac1p

Strain	Plasmid	0 Cu	+ Cu
w.t.	pYEX-BX		
	pYEX-BX-Vpr		
sac1	pYEX-BX		
	pYEX-BX-Vpr		

FIGURE 22

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[6] C07K 7/06, 7/08, 14/155, 14/16, 16/10; C07H 21/04; C12N 15/63, 15/48; C12Q 1/02; A61K 39/21, 39/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : FILE WPAT: Keywords: See below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC : C07K 7/06, 7/08, 7/10, 15/12, 15/28, C07H 21/04, C12N 15/63, 15/48, C12Q, 1/02

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
FILE WPAT: Keywords: VIRAL PROTEIN R, VIRAL PROTEIN X, VPR, VPX
FILE CASM: Keywords: as above and HIV or HUMAN IMMUNODEFICIENCY VIRUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO,A, 9200987 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 23 January 1992 See entire document; claim 9, 36, 46; page 4 line 26, pages 16 to 20	12-14
X	Journal of Experimental Medicine, Vol 172, september 1990, Choppin et al "Analysis of Physical Interactions between Peptides and HLA molecules and Application to the Detection of HIV-1 Antigenic Peptides" pages 889-899 See entire document; p 896 column 2 line 7; table 1 Vpr 68-80 (HFRIG)	1-7

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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		"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 1995	3 July 1995 (03.07.95)

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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	International Journal of Peptide and Protein Research, Volume 36, No 3, (1990) Gras-Masse et al "A synthetic protein corresponding to the entire vpr gene product from HIV-1 is recognised by antibodies from HIV-infected patients" pages 219-226 See entire document; Fig 1, page 221 column 1 paragraph 5 page 224	1-7
X	Journal of Virology, Volume 67 No 2 (1993) Lang et al "Importance of vpr infection of Rhesus Monkeys with Simian Immunodeficiency Virus" pages 902-912 See entire document; page 908, column 2 last paragraph	1-7
X	Journal of Virology, Vol 67 No 7 (1993) Balotta et al "Antisense Phosphorothioate Oligo deoxynucleotides targeted to the vpr gene inhibit HIV-1 replication in primary human macrophages" pages 4409-4414 See entire document	1-5, 8, 11, 16
X	WO,A, 9403596 (UNIVERSITY OF HAWAII) 17 February 1994 See entire document; claims 12 and 43	1-5, 8, 16
P,X	AU,A, 62524/94, (WEINER et al) 14 September 1994 See entire document; claims 2, 6, 14, 32	1-7, 10-14, 16-25
P,X	AU,A, 58487/94 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 29 August 1994 See entire document	12-14
P,X	Journal of Acquired Immune Deficiency Syndromes, Vol 7 (July 1994) Sarobe et al "Induction of neutralising antibodies against HIV-1 using synthetic peptide constructs containing an immunodominant T-Helper Cell determinant from vpr See entire document; abstract (HFRIG)	1-7, 12-14

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9015875	EP	474797	JP	4506605
WO	9200987	WO	9217945	US	5175872
AU	62524/94	IL	108707	WO	9419456
AU	58487/94	WO	9417825		

END OF ANNEX